

Answering the \$144 billion question

The US Congress will probably have settled the course of the world's economy for a decade in the past few days of hectic pre-Christmas legislation. It may not have the leisure in which to repent.

ONE thing about the future is now certain. The US federal government's deficit for the financial year beginning in October 1986, whimsically called "Fiscal 1987", will be not a penny more than \$144,000 million, just about two-thirds of the likely deficit in the current financial year. And how can anybody be sure of that in such an uncertain field? Because the Congress will this week have passed a law to that effect and because President Reagan will not have dared to veto the bill for fear that the federal government will otherwise be unable to borrow the funds it needs to pay its bills, pre-Christmas salary cheques due to federal employees among them. More than this, the Congress has also decreed a linear decline of the annual deficit until, in fiscal 1991, it vanishes altogether. It is as if a government had decreed that the incidence of murder, or of AIDS, will decrease in four equal instalments to vanishing point.

Naturally, neither the Congress nor the administration in Washington is as naive as these appearances suggest. The notion that the US government might first bind itself to make the deficit vanish and then buckle down to the task of reaching that previously unattainable goal has been around since the early summer. It has gathered force since September, when the members of President Reagan's political party in the US Senate felt themselves betrayed by the President's agreement with the Democratic Speaker of the House of Representatives which had the effect of killing off the Senate Republicans' plan for squaring the circle of the budget deficit. The notion provided marvellous material for newspaper columnists with opinions on the US constitution, for it means that Congress should surrender its right to legislate how public money is spent to a person whose only qualification for public administration is the possession of a hand calculator. But now, when the administration needs to increase its power to borrow money above $\$2 \times 10^{12}$ for the first time, the Gramm-Rudman bill (named after Republican senators from Texas and New Hampshire respectively) has inevitably attached itself to the bill that would provide the borrowing authority needed to keep the United States afloat. By now, President Reagan will probably have both his borrowing power and the threat that next year's budget will be determined not by priorities but by simple calculations of proportionality.

Basic research in the United States will be one of the early casualties. This is how that will come about. Next year's deficit has been pegged at \$144,000 million, and the administration has said that it will live within that limit. But the administration has also said that it will increase military spending by 3 per cent while not increasing taxes. Since large chunks of public spending (salaries and social security, for example) are fixed by law, the money will have to come from the government's dispensable spending, science and education included. This, no doubt, is why there is so much anguish about the preparations for the 1987 budget, due to be made public before February next year. It is hard to believe that the rising trend of research spending over the past five years will not be reversed next year, in the pursuit of a deficit of a mere \$144,000 million. But there may be worse to follow, for if the Congress and the administration fail to agree on a budget within the law, there will be *pro rata* reductions of all adjustable spending programmes. So next October, agencies such as the National Science Foundation and National Institutes of Health will be robbed of funds they had planned to spend, but

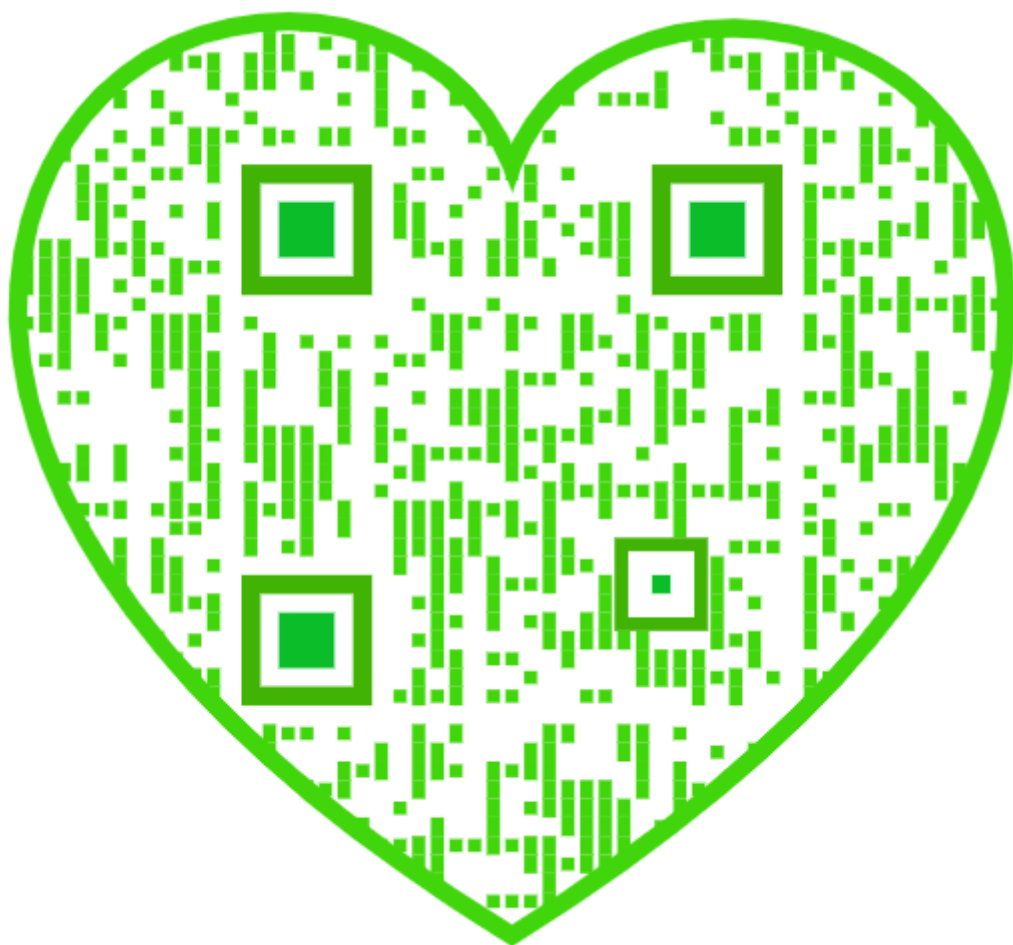
to a degree that will be known only a few days before the financial year begins. And there is an even more immediate threat; the same legislation specifies a cap for the budget deficit for the current financial year, now nearly a quarter gone. If, on 1 February next, it seems that spending will exceed \$176,000 million, which is almost certain, there will be an immediate cut across the board (which will nevertheless not exceed \$11,000 million). Will research grants be cut individually, or merely postponed? And with what effect?

The most helpful analogy of this way of conducting a great nation's financial business is the old recipe for extracting the teeth of dentist-shy children by tying a string to the tooth and to a door-handle, allowing the tooth to be extracted by the first person to open the door. In the last resort chance will be substituted for reason. It is a prescription for doing most damage to the public spending programmes which most of all depend on continuity and the pursuit of long-term objectives. It will be a great waste if the Reagan administration, with its creditable record of support for basic research over the past five years, should not put the enterprise into reverse, either in the process of meeting the targets Congress has decreed or in the inevitable ruckus about spending that is bound to erupt in 1986 (which is an election year).

Not that the objectives that the Congress had set for the government are invalid. The US budget deficit has been a literally mounting scandal for the past four years, causing endless damage in the United States and elsewhere. To cover its deficit while trying (successfully) to contain inflation, the US government has had to borrow funds that would otherwise have gone into private investment in the United States. So, until recently, the dollar has been overvalued relative to other currencies and the United States has been saddled with a deficit in its external trade numerically almost as large (\$140,000 million or so this year) as the budget deficit. As a consequence, the clamour had gone up that traditional industries being driven out of business should be protected against competition which is unfair only in the sense that it is caused by the budget deficit, and is thus artificial. As yet, the threat to world trade has been contained, largely because the administration has stoutly set its face against the erection of trade barriers, yet there is now alarming evidence that world trade is in serious trouble; OPEC cannot sell its oil, nor can the International Tin Council sustain the price of tin. So there is a danger that developing countries will be even less able to pay their debts by means of exports and that industrialized countries will further languish.

Gloom

If the gloomy forecasts that inflation has now given way to deflation should prove correct, it will then be plain that the Gramm-Rudman legislation has arrived at just the wrong time. For then, monetarists will be required to behave as if they were Keynesians, pumping money into the economy even if they have to print it, applauding budget deficits as the simplest way of curing an economic evil potentially as damaging as the inflation that seems only just to have been moderated. It seems inevitable that there must be some switch of policy along these lines long before the timetable for making the US budget deficit zero. Sadly, much damage has been done in getting this far. □



No pigs in pokes

A British dimension to the US Strategic Defense Initiative is not necessarily a bad thing.

THE trouble with the Strategic Defense Initiative (SDI) is that it means all things to all men. To Mr Reagan, it ensures immunity from other people's hostile missiles, but to Mr Gorbachev it is an abomination. Mrs Margaret Thatcher, the British Prime Minister, is somewhere in between. Through her, the British government has insisted that SDI is a research programme only, and that there is no question of deployment unless the owners of hostile missiles are consulted. Mrs Thatcher has also taken the lead in Western Europe in saying that influence of this kind requires collaboration, but only if the price is high enough.

How well does last week's agreement between the defence ministers of Britain and the United States match these good resolutions? Nobody can be sure. Virtually the only provision of the document made public is that which specifies that the substance of the agreement will be kept secret "in perpetuity". There will never be an opportunity for telling what costs, and what prizes the participants can expect. For all that can be told, the agreement may consist only of a single sheet of paper binding the participants merely to declare they have agreed to collaborate on SDI, but saying nothing else (together with the bit about perpetuity). So what should such an agreement contain? Even those who believe that Britain should not take part have an interest that the agreement should be equitable.

Technically, there are several important issues, of which the chief is whether SDI will be a stimulus to economic innovation. The argument is simple, but also simplistic. Spend money on military innovation, and you cannot fail to generate economically worthwhile innovations. So the more technical effort in British defence laboratories, the more prosperous British civil industry will become. There are two obvious objections. First, Japan, the most successful source of economic innovation, supports only a small military establishment. Second, Britain has for four decades spent conspicuously on defence research, but has been woefully inadequate at converting military into civil innovations. Whatever the secrets of last week's agreement, there is no chance that economic benefits will flow without structural changes in the relationship between military and civil research.

Worse, in British (but not American) conditions, the involvement of industry with powerful customers for knowledge (as SDI will be) can have the opposite effect; in retrospect, the dependence of the British telecommunications industry on the old public monopoly of British Telecom, far from making it competitive with free-standing industries elsewhere, actually made it harder for British companies to hold their own in world markets. At this stage, arguments about proprietary rights to inventions are academic.

So the long-term commercial benefits to Britain cannot be large unless there is structural change. Will the short-term cash (there has been talk of British organizations winning 7 per cent of the total) be of assistance? Here again, the arithmetic is clouded. If there is more money, some who might have emigrated may stay in Britain; the other side of that coin is that close acquaintance with SDI may tempt others who would have stayed in Britain to cross the Atlantic. But the most likely (and sombre) course of events is that great skill will be locked up in a programme of little economic value. The chances that people will pick up skills they never had, and will afterwards deploy them in civil applications, are small.

Technically, SDI is more likely to be a diversion than an opportunity. Politically, given the point from which the British government starts on strategic issues, it is on more sure ground. At some stage, there is bound to be an argument about the rights and wrongs of deployment. Most probably, if SDI yields anything of value to military technology (early warning is probably inevitable), there will be pressures to deploy what machines there are before the other side has been consulted. That is when

Mrs Thatcher or her political heirs will be able to cash the political cheques they will earn in the next few years. The question, and perhaps the darkest cloud over last week's agreement, is whether British governments will keep their independence and their nerve over the whole decade ahead. □

Australia's grudge

The McLelland report seems a fair complaint. What can be done?

THAT Australia should have been up in arms about the uses made by the British government of the deserts of Western Australia for testing British bombs three decades ago is natural and understandable. Last week's report on more than two years of a judicial investigation of the matter (strictly by a Royal Commission set up by the government of Australia) has failed to substantiate the more lurid tales told in evidence, but is otherwise fair. The chief recommendation, that the British government should somehow compensate Australia for the damage done, perhaps by cleaning up the radioactive mess left behind, is on the face of things a reasonable demand. But there is a danger that relations between the two governments will deteriorate into an unseemly squabble unless both of them acknowledge how much has changed, technically and in their political relations with each other, since the days when the Prime Minister of Australia, Sir Robert Menzies, was one of Sir Winston Churchill's closest buddies.

With the lapse of thirty years, it is hard fully to appreciate that even technical people could have been as naive as those in charge of the early nuclear tests about the dangers of radioactivity, both ionizing radiation produced directly from exploding fireballs and radioactive fission products or other radioactive isotopes produced indirectly, by the irradiation of material in the environment. To be sure, by 1945 there was a growing body of knowledge of the problems. The effects of X rays had been carefully studied, while the Manhattan project had thrown up a substantial understanding of the effects of external radiation by other materials, neutrons for example. The destruction of Hiroshima and Nagasaki eventually yielded crucial data about the biological effects of acute doses of external radiation. But of necessity, the effects of small doses (sustained by people some distance from the explosions) accumulated only as the years went by. So, by the time of the United States conference on the Peaceful Uses of Atomic Energy exactly thirty years ago, it was possible for the then chairman of the then US Atomic Energy Commission, Mr Lewis Strauss, to insist that there must be some kind of threshold dose of radiation below which no biological damage would be done. While Mr Strauss may have had a special flair for resisting unwelcome ideas, he was by no means untypical of those with influence in the 1950s. And only about that time did even the critics of nuclear weapons testing begin to accumulate telling evidence of the kind that eventually brought tests of nuclear weapons in the atmosphere to a halt.

The other important change that has come about in the past thirty years concerns Britain and Australia, then bound together both by the warm sense of having worked successfully together in a good cause (the Second World War) and, less easily, by the old ties of empire. It is hard now to reconstruct the familiarity of that relationship, although it seemed as natural to many Australians as it seems to have done to Menzies that the British should have been given more or less free range of the testing grounds in Australia. That the use they made of their licence may have been, by present standards, imprudent is amply demonstrated by last week's report. Much the same was happening then and earlier at the US testing grounds in the Pacific (see p. 502). The most urgent need now is that the British government should openly acknowledge the likelihood that much of Judge McLelland's argument is correct. To do that need be no shame (nor entail the A\$100 million talked of). To fail to make proper acknowledgement would be unjust, and make still more distant the relationship between Britain and Australia. □

SDI

Star wars research not up to scratch?

Victoria, British Columbia

THE US government is not rigorously applying the usual criteria for the assessment of scientific projects to all research proposals to the Strategic Defense Initiative (SDI). This is the nub of the allegations against SDI levelled by David Parnas, Landsdowne professor of computer science at the University of Victoria.

Parnas acquired a certain notoriety when, last June, he resigned from the computing panel of the SDI project, to which he had been appointed an adviser a

There's no money in crime—go for SDI contracts



few months earlier. He has since become one of the most outspoken critics of SDI on technical grounds.

Parnas is due to give evidence to a sub-committee of the US Senate's Armed Services Committee this week. He says that the essence of his complaint will be that scientists seeking research contracts have discovered that "when the decisions are made by people who have a mission, they tend to give the money to the people who make the biggest promises".

Parnas is especially critical of those at universities who have taken SDI money while knowing that the project will not succeed. His resignation letter in June said that he did not believe that "further work by the panel will be useful; I cannot, in good conscience, accept further payment for useless effort".

To the criticism (by SDI) that the computing problems would have been less conspicuous if those responsible for them had made greater use of artificial intelligence (AI), Parnas says that he has been watching the advocates of AI for years and has remained unconvinced. "They make extremely false claims. They solve very simple problems, but when you look at them closely, you find that the methods do not generalize."

To Parnas, the chief problems confronting SDI are that it will never be possible to test the system properly, while the researchers will not be able to define the be-

haviour of the attacking missiles with sufficient clarity.

In his own field, Parnas says that software is the last part of a system to which designers pay proper attention. Although it may be easy to "get the bugs out of" the mechanical part of a system, design flaws in the software will come to light only years after it has been put into operation. Yet the task facing the SDI software will be formidable; on one estimate, the system will need no fewer than 10 million lines of code.

Parnas guesses that, in an SDI system, there will have to be a computer on board every satellite, in the first instance to process the raw data. "Even if you are going to do the computing in a centralized system, you are going to have to do some

computing on the satellites to reduce the amount of data you have to transmit." Yet it will also be necessary for the satellites to control their own sensors and some weapons.

Parnas is convinced that the software needed for SDI is so complicated that it cannot be expected to function reliably. He argues that there will have to be systems for handling the data from the sensors, and for computing the trajectories of hostile warheads, distinguishing them from decoys. Then it will also be necessary to determine the paths of retaliatory missiles, and to decide when they should be fired. "Software", says Parnas, is the glue that holds such systems together." He notes that both the Apollo Moon programme and the space shuttle have been delayed by software problems. He is not against military research programmes, and remains a consultant for the US Navy. Yet he is resentful that money should be channelled into what he considers to be the wasteful SDI programme, as well as the wasteful use of manpower and of computers that will be involved. **Bill Johnstone**

SDI

British role is still unclear

Washington

THE long-awaited agreement signed last week in London on British participation in the Strategic Defense Initiative (SDI) should allow British researchers to bid for a "significant" proportion of the \$26,000 million the Reagan administration wants to spend on the programme over its first five years, according to British officials. But despite British efforts to secure a commitment from the United States to at least \$1,500 million-worth of contracts, no guarantees of specific sums are mentioned in the agreement.

British/US scientific collaborations have in the past been marred by accusations that Britain has failed to secure adequate returns. The most contentious aspects of last week's agreement, those relating to intellectual property rights, were not made public by British Secretary of State for Defence, Michael Heseltine, and US Secretary of Defense Caspar Weinberger, and the exact terms are likely to remain secret. Instead, Weinberger and Heseltine agreed that collaboration would be as "full and fair" as national laws permitted, and that there would be the "fullest possible retention of benefits of participation".

When dealing with domestic companies, the US Department of Defense (DoD) waives its rights to patents arising from basic research that it supports, with the proviso that it will not subsequently have to pay royalties to the developers. It is unclear whether it will adopt the same position for foreign companies.

Closely related to the question of intellectual property rights is that of security

classification. While the majority of SDI contracts let so far have been classified, those originating from the SDI organization's innovative science and technology office — some 5 per cent of the total by value — are unclassified, and the director of that office, James Ionson, has said that unclassified research projects will not be classified retrospectively. US specialists caution, however, that the limits to permissible commercialization of research funded by DoD are uncertain at the best of times, and depend greatly on how strictly the Pentagon chooses to enforce particular cases.

Another problem for British companies is likely to be that DoD as a matter of deliberate policy will often divide up or "compartmentalize" different sections of strategically sensitive research for security purposes; the result is to make integrating the results of a research project difficult for the individual investigator.

British officials stress that participation in SDI research does not imply an unfettered commitment to building and deploying a ballistic missile defence; Britain says it stands by an agreement reached a year ago between Prime Minister Margaret Thatcher and President Reagan, in which the two leaders agreed that any deployment of such a system would be subject to negotiations.

British participation in SDI will be coordinated by a new SDI participation office established within the Ministry of Defence in London. Areas where collaboration is expected include architectural design studies, optical computing and electronic materials. **Tim Beardsley**

French research planning

How will the future come about?

PERHAPS the French Revolution so divided and politicized the nation that every now and again France seems to need to pray together, to demonstrate its solidarity. This is the spirit in which researchers and industrialists met at the National Colloquium on science and technology in 1982, and this was also the spirit in which the same groups met again at the end of last month, at another grand mass — one praying for the future.

The meeting, "Prospective 2005", was jointly sponsored by the Centre National de la Recherche Scientifique (CNRS) and by the Commissariat du Plan (le Plan for short), which together had spent a year on the preparations. In many ways, the meeting marked le Plan's return to the public stage. Founded after the Second World War, le Plan became famous for its planning of France's spectacular economic rebirth during the 1950s. More recently, it has been somewhat moribund, suffering an uneasy relationship with, and neglect by the socialist government until, it seems, le Plan discovered technology.

The main fruit of the discovery so far seems to have been last month's colloquium on technological France twenty years ahead. But, unfortunately for le Plan, the general feeling was that the year of study by expert groups, and the voluminous documents they presented to the colloquium, amounted to a flop.

A report on communications, for example, was described by one communicator as "arid"; no doubt he was as aware as the other participants of the tremendous political debate then (and still) raging outside on the prospect of a fifth (private) French television channel run in collaboration with Italian and British interests and the future use of the first (and French) European direct broadcasting satellite to be launched next October.

The formal documents on the future of medicine similarly failed to take much account of the potential contribution of genetic engineering, but one of the colloquium rapporteurs dismissed — with venom — the notion that microgravity (as in a space station) might be of great importance in materials processing.

The rights and wrongs of these issues are certainly arguable. The future of French television was firmly outside the meeting's terms of reference. Less glamorous techniques than genetic engineering (such as halting smoking) might have more impact on public health in 2005 than any monoclonal magic bullet. But materials processing in space does occupy a large proportion of French research ministry funds devoted to work of this kind. One aggrieved representative of French industry complained at the meeting that the research ministry's funds devoted to space-based and land-based special prog-

rammes in materials science are in the ratio 4:3 (FF80 million against FF60 million). One consequence of this vein of realism, as the French would call it, was the opposite of what might have been expected of the nation that nurtured Jules Verne: the colloquium made the future seem a little dull.

The exception was the dramatic presentation of the future of unemployment in 2005. We are moving away from a civilization of pain and labour (*civilization de la peine*), the colloquium was told, to one of *civilization de la panne* (implying breakdown), a more flexible way of working than at present. The mere labour of the workers would no longer be needed, the colloquium was told; twenty years from

now, there would be 100,000 robots at work in factories, 25 times as many as at present. Before then (ten years from now), there would be an electronic workstation on every office desk. By 2005, retraining would occupy 10 per cent of people's working hours, which would by then be reduced by a quarter. The urgent need, the conference was told, is that institutions and trades unions should worry about the career patterns of the future.

This uplifting theme evoked a sour note. The intended massive increase of education and of retraining is a policy "of the elite for the elite" according to one who should know, the director of an employment office in north Paris with 3,500 unemployed people on its books. He doubted whether ordinary people would be able to cope with what the future, even as described, would expect of them.

Robert Walgate

UK medical research

MRC defends core of excellence

UNDER continuing financial pressure, the UK Medical Research Council (MRC) intends fully to protect the best jewels in its crown, but to throw out those that are substandard. Speaking in London last week, Sir James Gowans, secretary of MRC, said that only then would it be possible for the council to afford much needed equipment for existing units, to start new units and to give proper support to university research.

One unit to be backed up to the hilt is the Laboratory of Molecular Biology at Cambridge. A search committee has been set up to find a director to replace Sydney Brenner, who will be retiring from the post by 1987 and Sir James said that the

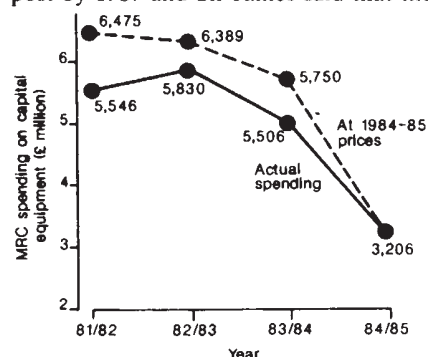
headed by Sir Michael Stoker that is due within the next two weeks.

Although MRC is well off relative to the other British research councils, with a budget that is falling only slightly in real terms, it has such big inescapable costs — mostly permanent scientific staff — that grants to university researchers, spending on capital equipment and the number of research studentships awarded have all fallen markedly in the past two years. For example, capital equipment spending has dropped from £6 million in 1982–83 to £3 million in 1984–85. Of projects worth £4 million rated to be of high priority for which central funds were sought this year, only £2 million could be found, although some more will follow from the extra £2.5 million recently added to the science vote.

Similarly, whereas it was possible in 1982–83 for MRC to support 80 per cent of the alpha-graded grant requests from universities, the figure has now fallen to 55 per cent. Most worrying, says Gowans, is that the total number of applications has fallen from 1,200 to 950 in the same period, suggesting that the scientific community is losing hope.

Unconventional ways of funding have been used for MRC's two current major initiatives. For the Institute of Molecular Medicine in Oxford, both the Wolfson Foundation and the Edward Penley Abraham Research Fund have contributed substantially to capital costs, and the research groups within the institute will be expected to compete for grants rather than be tenured. And the Centre for Collaborative Research, which will occupy a building being vacated by the Imperial Cancer Research Fund next to the National Institute for Medical Research at Mill Hill in London, is expected rapidly to become self-financing as a result of industrial backing.

Peter Newmark



council will have "some flexibility" in the salary that can be offered, although it will not be possible to compete with US universities or industry.

On the other hand, the MRC Pneumoconiosis Unit at Penarth has been closed because the science was not good enough and the Trauma Unit in Manchester is closing because it proved impossible to find a new director of sufficient calibre. Moreover, it is rumoured that two neuroscience units will soon be closed down, while the Clinical Research Centre must be nervous about the report of a committee

Polish universities

Government admits sackings

THE Polish government has now admitted that more than seventy university and higher college rectors, proctors and deans and department heads have lost their administrative posts, almost twice as many as was first reported.

An official announcement last Friday explained that under the amendments to the Higher Education Act, passed last July, the Minister of Science and Higher Education has the right to "review" senior university staff, not only on the basis of their academic work, but also of their "civic attitude". By the end of last month, university officials whose work or "civic attitude" was unsatisfactory had to be informed that they would not be confirmed in the offices to which they had been elected before the change in the law.

The dismissals have been interpreted throughout Poland as a move to regain some of the government and party control over the universities lost during the "liberalization" of the Solidarity era. This is, to some extent, confirmed by the official announcement which stresses that universities must be run according to what it calls the "state and socialist character" of higher education. Throughout Poland, students and academics have been vocal in condemning the changes, and at least one rector not himself affected by the dismissals, Dr Kazimierz Szewiotko of the Poznan Agricultural Academy, is said to have

resigned in sympathy.

Concern has also been expressed elsewhere. In London last week, the Polish ambassador, Mr Stefan Staniszewski, was summoned to the Foreign Office for what was officially described as a "sharp" exchange of views with the Minister of State Malcolm Rifkind. Mr Rifkind warned the ambassador that the dismissals were bound to have an adverse effect on British parliamentary and public opinion, and said that they did not sit well with official Polish assurances that normalization and reconciliation were proceeding satisfactorily in Poland. Mr Staniszewski is said to have expressed surprise that the British government should interest itself in an internal Polish matter, and said that the British government should stick to its 1981 stance that internal developments in Poland were for the Polish people to settle. Mr Rifkind replied that an expression of concern did not constitute interference, but that the dismissals made the Polish government claims seem rather hollow.

Mr Staniszewski himself departed from his stance of non-interference by observing that those concerned in the changes had not lost their teaching jobs whereas in Britain thousands of university lecturers were out of work. In the Polish universities, however, there seems to be a consensus that the dismissal of the officials is only the first step.

Vera Rich

Nobel peace prize

Controversy over the winner

THE 1985 Nobel peace prize ceremony this week was surrounded by last-minute controversy over one of the two persons delegated to receive it, Dr Evgenii Chazov, Deputy Health Minister of the Soviet Union.

The prize was awarded to the organization International Physicians against Nuclear War. In October, when the prize was announced, it was stated that two officials of the organization would represent it at the ceremony in Oslo, Dr Bernard Lown from the United States and Dr Chazov from the Soviet Union. The trouble started, late in November, when it became widely realized that Dr Chavoz had, in 1973, signed a letter from Soviet scientists attacking Academician Andrei Sakharov, who himself received the Nobel peace prize in 1975. Some human rights groups called for the 1985 prize to be withdrawn, and one group organized an "alternative peace prize" ceremony in honour of Dr Anatoli Koryagin, now serving a 7-year labour camp sentence for having published in *The Lancet* in 1981 a detailed study of the political misuse of psychiatry in the Soviet Union.

Dr Chazov's co-recipient, Dr Lown, has

been vocal in his defence. Dr Chazov, he says, has said nothing against Sakharov "since 1980". That was the year when the International Physicians against Nuclear War was founded, and also the year in which Dr Sakharov was exiled to Gor'kii. It is not clear which of these events (of both) Dr Lown had in mind. In fact, as recently as last week, Dr Chazov, speaking on the Norwegian ARD-Tagesthemen news programme, said that he was "not in agreement" with some of Sakharov's statements on the nuclear arms race, and quoted an unnamed US physicist to the effect that Sakharov was being made "a prawn in the cold war" and exploited "in a pharisaical manner" in pursuit of anti-communist aims.

Dr Lown, however, seems to have had considerable difficulty with his media statements. In particular, he was reported to have told the wire services that he considered it "shameful" that the US ambassador to Norway had decided not to attend the award ceremony, and then, several hours later, to have told the BBC's "World At One" programme that this was the first he had heard of the ambassador's decision.

Vera Rich

US defence contracting

NASA chief steps aside

Washington

THE US Army's Sergeant York anti-aircraft gun, whose development was cancelled in August after \$1,800 million had been spent on it, seems more effective at damaging people than aircraft. Last week, Mr James H. Beggs, administrator of the National Aeronautics and Space Administration (NASA) for the past four years, was forced to take indefinite leave of absence after being indicted by a Los Angeles grand jury for complicity in an alleged fraud by General Dynamics on the US government.

General Dynamics, best known as the constructor of the *Trident* submarine and the F16 fighter aircraft, has been one of several military contractors in trouble with the Department of Defense over cost accounting, and was suspended from receiving US government contracts for a period earlier in the year. Mr Beggs was a vice-president of General Dynamics, and the one in charge of the company's brief involvement with the anti-aircraft gun until he moved to NASA.

This unexpected development is bound to be a setback for the agency. It seems to be agreed that the year ahead will decide how quickly, if at all, NASA secures the funds with which to build the permanent space station on which NASA has set its heart.

Against the background of a probable budget squeeze early in the new year, this is also a time when an agency cannot easily afford to lose its chief administrator for an extended period.

Both Beggs and General Dynamics hotly deny the charges levelled against them, which centre on allegations that the company, while working on a fixed-price US Army contract to make prototypes of the Sergeant York gun, charged excess costs on the project against other military contracts.

The amount involved is alleged to have been \$7.5 million in a total contract price of \$40 million. On the basis of the prototypes, the development contract was eventually awarded to the other bidder, Ford.

At no point does the Los Angeles indictment suggest that the alleged fraud was other than for the benefit of the company. Three other officials of General Dynamics have been indicted, together with the company itself. Company spokesmen have been suggesting that the origin of the charges lies in the procedures used for charging particular costs to various contracts.

The length of Begg's absence from NASA is undetermined, but will presumably be at least as long as the time it takes for the case to come to trial. □

British pharmaceuticals

Spin-off for research

THE Wellcome Foundation, the British pharmaceutical manufacturer, last week announced its best results ever. This development may have important consequences for British medical research because the company's sole shareholder, the charitable Wellcome Trust, already a substantial source of research funds, plans to sell 20 per cent of its major assets early in the new year. The higher this year's profits, the higher next year's share price is likely to be.

Wellcome's annual report for 1985 says that an increasing share of its business now derives from the United States, and that last year's profit of £121.7 million is largely accounted for by the then favourable exchange rate between the dollar and sterling. Even so, profits are up by 37 per cent over last year, and amount to 12 per cent of the turnover of £1,004 million. Thanks to aggressive marketing, the US business accounted for 45 per cent of turnover and 73 per cent of the profit during 1985. The company spent £122 million on research and development last year.

For the year ahead, the pharmaceutical business hopes to do well with innovations prompted by concern over AIDS (acquired immune deficiency syndrome). Wellcome has to decide soon whether to seek to market its diagnostic tests for AIDS in the United States; the test is one of two selected by the Public Health Service in Britain for the routine screening of all donated blood, which began a few months ago.

Through its US subsidiary, Burroughs-Wellcome, the company is also hoping to make some headway with the antiviral compound azidothymidine, otherwise known as AZT, which is said to inhibit the multiplication of HTLV-III (human T-lymphotropic virus type III) in cell cultures and to have done well in toxicity tests in human subjects. Wellcome says that the drug development process has been shortened from three years to three months in the development of AZT so far, which is a measure of the eagerness of drug houses to put an anti-AIDS drug on the market. But Wellcome is exceedingly cautious about the potential of AZT, no doubt so as to avoid embarrassment of the kind following last month's announcement from Paris of a "successful" treatment of AIDS (see *Nature* 318, 3; 1985).

The sale of 20 per cent of Wellcome's assets in January 1986 is expected to raise £300 million for the trust. The trust will then spend an extra £10 million per year on the support of permanent medical research groups in British universities (see *Nature* 317, 662; 1985), which, added to its present expenditure of £20 million, will make it the single largest supporter of such research in the United Kingdom, spending more on medical research in the uni-

versities than does Britain's Medical Research Council.

The trust is still trying to decide how to attract the best projects. Last week it announced an award to Professor Neil Brooks and Dr James McCulloch at the University of Glasgow for the study of Alzheimer's disease. The research will be

Advanced ceramics

Rush to metal/oxide composites

Boston

A NOVEL method of making ceramic metal composites, known as the lanxide process, was a major attraction at this year's autumn meeting of the Materials Research Society in Boston. The new process, rumoured for some months, was described in public for the first time by Dr Mike Newkirk of the Lanxide Corporation of Newark, Delaware. It promises new tough ceramic composites at significantly lower cost than existing methods, which tend to be expensive and produce a brittle end result.

Lanxides are formed by reaction between a molten metal and a vapour-phase oxidant, for which air will suffice. Typically, the metal has to be doped with a least two dopants — magnesium and silicon work for aluminium — and the temperature of the melt brought to within set limits (1,250°C in this example). The lanxide, in this case a coherent composite of aluminium and interconnected aluminium oxide, forms at the metal surface.

The mechanism of the reaction remains obscure. The material grows from the metal/oxidant interface towards the oxidant, and metal is transported through the growing lanxide by a process that appears not to be reliant on diffusion. The properties of the material, which can be grown in slabs an inch thick can be adjusted by altering the temperature of the melt and by depleting (or not) the reservoir of molten metal.

The microstructure, which reveals a millimetre-scale columnar grain, changes over the cross-section of the lanxide. By appropriate choice of conditions, tensile strength or toughness of an aluminium/aluminium oxide lanxide can be increased significantly above that of sintered alumina. No details of other lanxide materials were given last week, but Newkirk said that "hundreds" of inventions arising from the new technology are being patented.

Military and proprietary interest in the new materials is intense. Obvious applications include rocket and jet engines, armour plating and the Strategic Defense Initiative, where there will certainly be a demand for strong but light materials. According to Professor Rustum Roy of Pennsylvania State University, the im-

ported for at least 10 years and will cost about £1.3 million. And this week the trust is advertising a new award of up to £3 million to establish a research group using non-invasive methods to study the brain. Much of this money will be used to buy instruments such as nuclear magnetic resonance machines, normally too expensive for researchers in the United Kingdom. The trust expects to appoint the successful applicant in late 1986.

Maxine Clarke

portance of the process was brought to the attention of President Reagan soon after it became apparent, and much of the development has been funded by the Defense Advanced Research Projects Agency under conditions of strict secrecy, with arms traffic regulations being invoked to restrict access of technical details to foreigners. Earlier this year, Lanxide Corporation, co-founded by Newkirk two years ago to commercialize the process, received an \$18 million cash injection from Alcan Aluminum Limited in exchange for a 42 per cent holding. Joint ventures between Lanxide and other companies are now also being negotiated.

Tim Beardsley

Bad day for Yonas

THINGS did not go too well last week for Dr Gerold Yonas, chief scientist of the Strategic Defense Initiative (SDI) Organization, when he addressed the Materials Research Society on "Materials for SDI".

Reliability of complex systems has surfaced as a potentially major problem for SDI, and half of the organization's budget is devoted to battle management systems and communications and control. But the frailty of even the most carefully staged events was emphasized when Yonas got up to speak, only to find himself completely inaudible to the audience of close on a thousand. Conference aides fussed and fumbled for a couple of minutes before it was discovered that someone had forgotten to switch on Yonas's microphone. The irony was lost on nobody.

Yonas proceeded to gallop through his usual one-hour presentation on SDI, spiced with some new references to the need for strong and light materials in, for example, space-based reflectors for lasers, and for materials with low coefficients of expansion in infrared lasers. The talk would have been better suited to an audience of high-school students or perhaps congressmen than to a technically-sophisticated group of researchers, and the applause was less than enthusiastic. After some generally hostile questions, Yonas left, perhaps wishing he had not bothered to come.

Tim Beardsley

Information technology

Britain's flagship looks for help

FLAGSHIP, an optimistic collaborative project between the British government, industry and two university departments, was launched in London last week. A £9.3 million investment is being made by the Department of Trade and Industry's Alvey programme with a consortium comprising ICL (Britain's only major computer company), Plessey, Imperial College London and Manchester University, increasing to £15.5 million for the initial three years of the project. Flagship is the long-awaited fifth-generation information processing system with many general-purpose applications. But whether the British project can keep up with Japanese and US efforts is open to question. Certainly legal squabbles over who is to reap the profits from products developed by Flagship have delayed the start of the project.

The general aim of Flagship is to evolve an architecture with sophisticated parallel hardware and "intelligent" software that will be easier for the non-technical user to operate. Imperial College has developed the software for Flagship (the Alice graph-reduction machine) and the hardware is provided by Manchester University's Dataflow machine. ICL is to develop the system at its Manchester site. The Alice prototype uses Inmos transputer chips, but ICL says that recent worries about the future of Inmos will not affect Flagship —

"we already have all the transputers we need".

The Flagship project will concentrate on declarative styles of programming, and will use Dactl, Alvey's compiler target language, which Alvey hopes will be adopted as a European standard. Flagship also uses Pisa (persistent information space architecture) for interfaces between applications, computers and data.

The Alvey programme was set up in 1983 to stimulate research on advanced information technology; its priorities are intelligent knowledge-based systems, man-machine interfaces, software engineering and very large-scale integration. The aims of fifth-generation computers were set out by Japan in 1982; such an architecture should produce computer

systems that work 1,000 times faster and handle 10,000 times more information than existing machines, as well as the "social" function of communication in a natural language rather than machine codes.

The applications of the Flagship type of system are enormous — project management, "knowledge" databases, image processing, robotics and office systems are some areas that Flagship will be seeking to develop in the next few years. The biggest threat to Britain's commercial aspirations is seen to be from the Japanese, who are putting considerable effort into advanced systems architectures. For example, Japan supports eight giant electronic corporations that make microchips, compared with Britain's single troubled company, Inmos. At Flagship's launch last week, Mr Geoffrey Pattie, minister for information technology, hinted that Britain might look for European collaboration for future stages of the project. **Maxine Clarke**

European Synchrotron Radiation Facility

Grenoble siting hits a snag

THE Prime Minister of France, M. Laurent Fabius, may have broken the law when he decided to site the European Synchrotron Radiation Facility (ESRF) at Grenoble rather than Strasbourg. That at least is the opinion of M. Jean Raymond, an adviser to the local government of Strasbourg, who is planning to bring the issue to a head this week. M. Fabius has only just emerged, apparently unscathed, from the trouble caused by his remark last week that he had been "troubled" by his president's reception of General Jarulewski at the Elysée Palace.

M. Raymond's case against the Prime Minister was argued at a public meeting in Strasbourg a week ago, and is based on an agreement between the national government and that of the Alsace regional council which was signed in April 1984. The national government apparently agreed to "support the candidature of Strasbourg as a site for new international organizations". It supplied a list of eligible organizations, among which ESRF was one.

One question is whether all the items on the list were to be considered for Strasbourg in the first instance, or whether they were merely examples; Raymond believes the intention was clearly the former. But the second more substantial question is whether the agreement can be legally binding whatever interpretation is correct. Raymond argues that planning laws enacted in 1982 and designed to devolve power to the French regions do indeed have the force of law. He seems to have a point when he adds that none of the procedures specified in the 1982 act for rescinding agreements between central and local governments seems to have been followed when ESRF was moved to Grenoble.

A dispute between the regional government, which belongs to the political right,

and the socialist government in Paris thus seems inevitable. M. Fabius's case would undoubtedly be stronger if the machine intended for Grenoble were already partly built. But as yet, no ground has been broken there; indeed, the government is still seeking foreign contributions towards the project. So the return of ESRF to Strasbourg is not impossible.

Robert Walgate

UNESCO minus one

THE British government last week formally announced its resignation from UNESCO (United Nations Educational, Scientific and Cultural Organization), to the consternation of many remaining members. The £6.4 million that Britain contributed will be transferred to the British Council, Mr Timothy Raison, minister for overseas development, said last week. The government will use the money to enable more students from the developing countries to study in Britain; numbers are expected to increase from 11,000 to 12,000.

Members of other European Community countries, particularly France and West Germany, Japan and members of parliament from the three major parties in Britain, all objected strongly to Britain's decision to withdraw. Mr Amadou Mahtar M'Bow, UNESCO's director general, expressed his "deep regret" at Britain's departure.

Britain, after the departure from UNESCO by the United States last year, had demanded many reforms in the organization (see *Nature* 14 November, p.98; 5 December, p.397). Some were implemented at the recent general assembly in Sofia, but Britain felt that UNESCO had not gone far enough.

Maxine Clarke

Satellite insurance

ARIANESPACE, the French-based company that sells space on the European space launcher Ariane, has moved quickly to offer customers insurance at reasonable cost (see *Nature* 317, 567; 1985). The company has set up a subsidiary, the Société de Réassurance des Risques Spatiales (known as S3R), which will offer users a free re-launch of a failed satellite.

S3R is designed to cover the next fifteen Ariane launches over the next five years, and will offer insurance at premiums of 10–13 per cent, compared with the 20–30 per cent being quoted unofficially by the open markets. The difference stems from S3R's sanguine view of the risks. It expects only one failure in the next fifteen Ariane launches, compared with the one-in-five risk expected by private insurers.

Even so, the new company expects to lay off some of its risks on the private re-insurance market. S3R is meant only to cover the next three years, after which it is hoped the insurance market will have been reassured by Ariane's performance.

Whether the terms now offered constitute a subsidy will be fiercely argued both by the private insurers and by those who regard Ariane as a serious competitor of the space shuttle. **Robert Walgate**

US bomb tests

Radiation tests underestimated?

Washington

THE US Department of Defense (DoD) was criticized last week by the General Accounting Office (GAO) for underestimating the exposure to radiation of 42,000 service veterans who participated in the 1946 "Operation Crossroads" atomic weapons tests at Bikini Atoll. According to GAO, the DoD's assessment, on which basis the Veterans Administration has so far rejected medical claims based on radiation exposure, may underestimate or entirely overlook significant beta exposure, while the internal alpha dose may be underestimated by a factor of 5 to 10.

The two Crossroads explosions, the first above water and the second below, were the first atmospheric weapons tests in peacetime, and radiological protection procedures were crude or non-existent. Decontamination procedures, showering and changing of clothes, for men working to clean up ships moored near the explosion, were only instituted six days after the second (and much dirtier) test, as the extent of contamination became apparent. The clean-up was eventually halted because of high radioactivity level, but only 6,300 of the 42,000 participants in Crossroads wore film badge dosimeters, and none wore badges every day.

Because records are so incomplete, DoD in 1977 asked its Defense Nuclear Agency to reconstruct exposures by extrapolation from known cases. The agency's conclusion, published last year, was that 93 per cent of those involved received exposures of less than 0.5 rem, and that all received less than 1.7 rem (the permitted total for radiation workers in the United States today is 5 rem per year). But GAO has identified several respects in which it believes DoD's analysis is in error.

Most doubt centres on the accuracy and interpretation of film badges. The badges did not work in the way expected, possibly

because of the effect of humidity, and GAO believes beta exposures could as a result have been seriously underestimated: 100 per cent error can be expected at the lower end of the range, for which DoD made no allowance. GAO also finds that personnel decontamination and protection procedures assumed by DoD were not in effect (immediately after the tests) or were widely ignored; in none of the photographs of the clean-up operation are the men wearing protective clothing.

DoD has formally disagreed with many of GAO's conclusion, but GAO retorts that DoD's comments are based on incor-

rect and unsupported statements and misinterpreted documents. GAO's recommendation is that DoD be required to revise its estimates to take account of the sources of error now identified. If the revisions are carried out and GAO's conclusions validated, the implications could be great for the 500 veterans' disability claims relating to Crossroads, as well as to the many thousands of others relating to different tests; the Veterans Administration has already indicated that it wants to extend GAO's findings. The GAO report, which was requested by Senator Alan Cranston of the Senate Veterans' Affairs Committee, will doubtless form the background to what may be expected to be stormy congressional hearings in the near future.

Tim Beardsley

Computers

Putting back the clock

Berkeley, California

CONVENTIONS held sacred for decades by the designers of computer hardware and software are being seriously challenged by computer researchers at the University of California, Berkeley. At the fore of the challenge is a new development in microchip technology which has simplified the design of the electronics needed for computers and jettisoned redundant and laborious machine coding.

That part of the Berkeley revolution began about five years ago, but its proponents believe the change is at last about to be accepted in the industry despite the controversy the ideas have generated and the hostility they have provoked. According to David Patterson, an associate professor of the Computer Science Division at Berkeley, and one of the creators of the new machines, "1986 will be the year when we see what happens".

The new computer designs were arrived at by inspecting a VAX minicomputer and identifying many machine code instructions that were never used in most com-

putational exercises. In that inspection, 304 such instructions were reduced to one tenth that number. So was born the Berkeley computer concept, called Reduced Instruction Set Computers (RISC).

The RISC machines turned the clock back in computer design. The evolution of computers — mainframe, mini and the micro designs in the 1970s and 1980s — used volumes of complicated instructions (the basic code programming the machines) and relied more and more on intricate and faster microelectronics to control that complex structure. A computer evolved within the computer, controlling these arrays of instruction codes, most of which were never used.

Berkeley's idea was to minimize the coded instructions needed to operate the machine and, at the same time, to design a simpler microchip. The RISC microchip made at Berkeley, the hub of the new machine, now has over 41,000 transistors and 60 32-bit registers. It can be fabricated on silicon down to a gate size of 4 µm with a speed of 500 ns per instruction.

Berkeley's research has been matched by that of others at Stanford University, IBM at Yorktown Heights and now at Hewlett Packard. Says Patterson, "Hewlett Packard is going to base its whole next generation on these ideas". IBM is rumoured to be developing workstations on the same principles.

Patterson is confident that RISC is a forward step despite its apparent resort to simpler technology. "Many people find this to be a ridiculously bad idea. It's a de-evolution of computer design... a dangerous concept. Shouldn't be talked about". Patterson and his Berkeley team are continuing to talk about it, and are developing the concept even further to see if the RISC machines can be further enhanced for the scientific and academic use of computers. One such application to be pursued is the use of RISC for artificial intelligence.

Bill Johnstone

Soviets admit AIDS cases

THE Soviet Union has now admitted that AIDS (acquired immune deficiency syndrome) has reached the Soviet Union. Interviewed in the newspaper *Sovetskaya Kultura*, Dr Viktor Zhandrov, a leading Soviet virologist, denied the growing rumours that there are "numerous" cases of AIDS in the Soviet Union but acknowledged that there had been an "insignificant number of cases" reported.

This comes less than two months after Soviet denials that there were any cases of AIDS in the Soviet Union (see *Nature* 317, 659; 1985).

Dr Zhandrov's article is less polemic in tone than previous Soviet statements on the disease, which stressed its connection in the West with drug addiction (relatively

rare in the Soviet Union) and homosexuality (which is illegal).

He stressed, moreover, that the disease seems to have originated in central African monkeys. This is in marked contrast to a recent trend in the Soviet media to attribute AIDS to United States bacteriological warfare experiments; although much of this reporting seems to be anecdotal (for example, that in Kenya AIDS has developed in proximity to a US base), not all of it has been directed at Third World audiences. In particular, on 30 October, *Literaturnaya Gazeta* quoted the Indian popular daily, *The Patriot*, to the effect that AIDS was developed in the United States at Fort Detrick as a weapon against the black and homosexual communities. Vera Rich

Japanese research

Private investment still growing

Tokyo

RESEARCH in Japan's private sector is booming as never before. Expenditure has grown 12 per cent this year (eight times as fast as the government science budget) with 78 per cent of the nation's research funds now coming from industry. These figures set the scene for the Science and Technology Agency (STA)'s white paper, *New developments in R & D and the age of cooperation*, which was approved by the cabinet last Friday.

The aim of the white paper is not so much to sing the praises of industry as to point out some weaknesses that in part stem from such a high reliance on the private sector and to suggest ways of correcting them. One new bill aimed at increasing cooperative research is all ready to go before the Diet.

Overall investment in research and development is growing proportionately faster than the economy and has now reached 2.8 per cent of national income, not far short of the 3 per cent goal set by the government. Virtually all companies involved in high-technology industries — electronics, telecommunications and biotechnology — are spending at least 8 per cent of their sales on research and development.

The other side of the coin is the relative weakness of government and university research and failure of budgets to keep up with inflation for several years running. This means that there are weaknesses in areas that industry cannot easily tackle; those requiring large-scale interdisciplinary cooperation and those requiring long-term cooperation for an uncertain pay-off.

Lack of government impetus partly reflects low government expenditure, and partly low levels of collaboration between the universities, government research institutes and industry. This is a perennial problem, but the need to get something done seems more urgent than before. Almost all advanced nations are pushing hard in the same high-technology fields. And as the report neatly points out, they are all adopting very similar science policies; everywhere industrial investment is being increased through similar packages of tax incentives and policy designed to spur cooperation between different research fields.

The white paper identifies key problems and, not surprisingly given that the paper is the work of a government agency, suggests solutions that nearly all involve increased expenditure at government laboratories. Two of the problems are interrelated; *per capita* expenditure on basic research is still low compared with Western nations, and basic research in public laboratories is insufficiently creative. The first is a problem of money. Unfortunately whatever STA might believe is correct,

there is little chance that the government will increase basic research funding when it has a fiscal deficit proportionately larger than that of the United States. The second is more a problem of organization and in particular failure to provide young researchers with the freedom to put their talents properly to work. The problem is now to be taken up by the Council for Science and Technology — Japan's highest advisory body chaired by the Prime Minister. A few days ago, the council agreed to a wide-ranging debate on the operational management of the public research institutes.

A third problem seems to stem from a feeling that anything the West is doing Japan should be doing too, in this case its giant research projects such as the Strategic Defense Initiative (SDI) and the emerging Eureka programme which might produce a quantum jump in technological level. There is nothing really new on the horizon, to follow large-scale projects such as the development of the

"bullet" train and automatic telephone switching gear. STA officials suggested that international cooperative projects in cancer research, fusion energy, giant accelerators and space platforms might all be candidates for the development of completely new technologies. But there is little prospect of a major publicly-funded Japanese research initiative under current budgetary constraints.

Positive action is at hand to deal with the fourth problem, that of encouraging cooperative research between industry, government and the universities. The need for some form of cooperative research is obvious to industry: in the past few years, there has been a striking rise in joint research performed abroad. Growth in tie-ups with domestic institutions has been much more sluggish. A new bill to be introduced by the cabinet in March and intended to clear the Diet by the summer should remove at least the legal restraints on cooperation between the public and the private sectors. The bill will also make it easier to carry out cooperative research with foreign companies and tackle problems of patent rights. **Alun Anderson**

CEGB

Acid rain storm from Norway

"UNBALANCED and biased", Norwegian Ministry of the Environment official Jan Thompson said of the British Central Electricity Generating Board (CEGB) video "Acid Rain". The Norwegians pulled few punches at the press conference they had called in London on Monday of this week to give their response to the recently released film. Thompson continued "the views put forward in the CEGB film are obsolete and dangerous . . . and will in the long run strain the relationship between the two countries. A cynical attitude was expressed".

The 20-minute video, widely distributed throughout the United Kingdom, purports to present a balanced and objective view of the acid rain issue, and concludes that the effects on the environment of acid precipitation may have been overestimated and that there is considerable doubt in the scientific community that the widely reported deaths of fish and forests in Europe are caused by acid rain. In addition to CEGB personnel, the film uses interviews with Norwegian, Swedish and German scientists, and was partly shot in Norway. The Norwegians say that neither their environmental authorities nor major research institutes were notified. They also claim that there was considerable bias in the selection of persons interviewed, and that interviews were cut so as to present a distorted view of opinions expressed. At least one scientist, Swede Hans Hultberg, is understood to have protested strongly against the editing of his comments.

The seriousness with which the Norwe-

gians are treating the affair can be judged from the fact that in addition to a report on the video sent to CEGB, a letter from the Norwegian Prime Minister, Kaare Willoch, has been delivered to Mrs Margaret Thatcher. The subject of acid rain was prominent in a meeting between the two late last month. In carefully chosen words, Jan Thompson said "we are not talking about a diplomatic protest".

The Norwegian scientists at the conference — Professor Seip and Dr Muniz from the University of Oslo and Mr Dovland from the Norwegian Institute for Air Research — presented detailed evidence, some new but much previously published, to support their case that some 15–30 per cent of the sulphur deposition in southern Norway originates in the United Kingdom. They argue that the recent reductions in emissions from Britain result only in fine tuning when used in long-range pollutant transport models. The claim is not that stopping emissions from Britain alone would solve the problem, but that concerted European action is needed to stop this "chemical war", as Muniz described it.

The Norwegians claimed that the natural environment is being seriously damaged and that it is disturbing that acid precipitation can be so lightly dismissed by CEGB on the grounds, among others, that 100 per cent scientific certainty has not yet been achieved. Borrowing a symbol for the *Bulletin of the Atomic Scientists*, Seip showed an acid rain clock; the hands stood at five minutes to midnight.

Peter Gambles

Dioxin exposure at Monsanto

SIR—Hay and Silbergeld urge a re-examination of the information about workers exposed to dioxin at the Monsanto plant in Nitro, West Virginia¹. They do not actually challenge the conclusions of two studies that there were no excesses of cancer or heart disease deaths among dioxin-exposed workers, but they convey the impression that the studies are seriously flawed.

In particular, they draw attention to the fact that four men classified as exposed to dioxin in one paper were classified as not exposed in the other. The first paper, which classified the men as exposed, defined exposure by the presence or history of chloracne², a disease known to be associated with dioxin. The other, which classified the men as not exposed, defined exposure by a history of work assignments in processes involving dioxin³. Neither measure is perfect, and they are not congruent. Although the differing classifications are disquieting, at least three of the cancer deaths are probably independent of dioxin exposure. Those three were lung cancer deaths among smokers; the fourth was from Hodgkin's disease, for which there is no strong evidence for association with dioxin. If the differing classifications for the four men are enough to destroy the conclusions reached by the authors of the studies, that analysis should be developed by Hay and Silbergeld.

From Monsanto records that they do not further identify, Hay and Silbergeld state that 19 workers who would be classified as exposed and who died from circulatory disease or cancer were not included in the second mortality study. They do not provide information about how they determined the men's exposure, the number of unexposed men who died from the same causes during the same time period, nor when the men died. If they died before 1955, they would not have been included in the study, which, because of records limitations, was restricted to men who left the company after that time. A telephone call to the authors might have clarified the reason that those men were not included. Whatever the outcome of such a call, it would not have contributed to the aura of conspiracy achieved by the sentence "No reason is given for not including those cases".

Workers exposed as a result of the 1949 explosion at Nitro are generally considered to have been more heavily exposed than workers involved in usual processes at the plant. Hay and Silbergeld, referring to a 1953 study⁴, contend that the basis for that conclusion was more severe chloracne among the accident-exposed workers. While chloracne was worse among those workers, a panoply of other health complaints — fatigue, incapacitating aches and pains, libido and potency problems — was also elevated, lending cre-

dence to the contention that accident-exposed workers were more heavily exposed. I am uncertain what point Hay and Silbergeld want to make as they labour over the conjecture that there may have been no differences in exposure levels, but they follow that effort with criticism of the authors of the mortality studies for separating the two groups of men in their studies. The criticism is misplaced. While one paper² was limited to workers involved in the aftermath of the 1949 accident, the other³ included all hourly workers who terminated their employment after 1955, whether or not involved in the 1949 accident.

Having found no evidence of excess cancer in the Nitro workers, Hay and Silbergeld turn the idea of dose-response relationships on its head and suggest that diseases such as cancer "may be expected" in people exposed to "lower and more chronic exposures". References offered in support of that idea are two books that contain data both suggesting and not suggesting a link between dioxin and human cancer and two papers associating cancer in herbicide sprayers with dioxin. The herbicide sprayers characterized as chronically exposed were exposed only during one or two months a year⁵, for a median total exposure time of 42 days. Studies of another population, which included more than 1,500 sprayers who had been exposed for at least 12 months, found no cancer excess⁶. Furthermore, some Nitro employees worked for years in production processes that involved dioxin and some, not exposed through the explosion, developed chloracne⁷. Those men were surely chronically exposed to dioxin, many were included in the Zack and Gaffey³ study, and there is no cancer excess. The hypothesis that low-level, intermittent exposures bear more risk than higher-level exposures requires substantiation.

Hay and Silbergeld's penultimate paragraph refers to a health survey⁸ as support of a possible connection between dioxin and an "excess of cardiovascular-related deaths". The health survey presents no new data about mortality; it does refer to an excess of cardiovascular deaths in Monsanto workers already reported by Zack and Gaffey³. An editorial that accompanied publication of the health survey suggested that the health survey might include some evidence for an excess of myocardial infarctions (not deaths) among men who had had chloracne⁷, but that the appropriate analysis is wanting. Finally, the mortality study that reported the excess of cardiovascular-related deaths in Monsanto workers found no difference in death rates from the cause between workers exposed and not exposed to dioxin¹, and the number of cardiovascular deaths among chloracne-stricken workers was less than 75 per cent of the

expected number⁷.

Hay and Silbergeld criticize studies that use chloracne as the criterion of exposure. They are on firm ground when they say that the disease is a dependent variable of exposure and that epidemiological studies usually use some independent measure, such as work histories, as the criterion for exposure. However, they raise no criticism of the health survey⁸ for depending on chloracne as a measure of exposure. Apparently, a double standard is used, studies that cannot be interpreted to show excess deaths being held to a higher one.

In Hay and Silbergeld's words, "the epidemiological picture at Monsanto remains confused". The picture is more unclear than anyone would desire, but it is not quite so confused as they have it. Their letter shows that it is possible to locate flaws and select studies to support an argument that dioxin is possibly a scourge. Equally, it is possible to read the literature and arrive at a cautious conclusion that dioxin has not caused early death and cancer in heavily exposed workers. That conclusion, which is widely accepted⁸ and draws on information from dozens of studies done in the past decade, might lift some anxiety from people who have been environmentally exposed to lower levels of dioxin.

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AIDS

SIR—Misinformation on acquired immune deficiency syndrome (AIDS) continues to abound and Ian Davidson's comment (*Nature* **317**, 666; 1985) is another example.

It is wrong on two counts: (1) It is perfectly possible to be promiscuous and have little risk of contracting AIDS, providing one avoids certain high-risk sexual practices; (2) Non-promiscuity does not guarantee that one will not contract AIDS. Certain groups are still at risk, for example wives of gay or bisexual men, and intravenous drug abusers.

I do not know if chastity has replaced death as the "great unmentionable", but fiction seems to have replaced fact when it comes to the truth about AIDS.

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Whose rings around Neptune?

What seems to be an authentic and interesting discovery of an incomplete ring around the planet Neptune seems certain to be published piecemeal for lack of agreement among those concerned.

READERS will be puzzled by the tentative explanation of the partial ring around Neptune provided on page 544 of this issue by Dr J. Lissauer of the University of California, Berkeley, chiefly because they have no reason to believe that there is such a structure around this distant planet. Surely, readers will ask, it will be time enough for Dr Lissauer to produce his explanation when there is evidence to suggest the need for one. And why should this apparently pointless explanation begin with the flat unsubstantiated declaration that "an incomplete arc system has recently been discovered around Neptune"?

Dr Lissauer should not be judged too harshly. Evidence for a partial ring system around Neptune does indeed exist. Roughly six months ago, an article giving a full and interesting account of the discovery was submitted to *Nature* for publication, was accepted for publication and would long since have been published had not those responsible for the collection of the evidence fallen out over the wording of the paper. All the authors have throughout expressed distress at being caught up in such a childish business yet have been unable to reconcile their differences.

What follows is an account of the circumstances leading to this sad state of affairs, from which two lessons may be learned. First, there may be occasions when pride takes precedence over the principle that science is an open process, in which publication is a duty owed to the rest of the community. Second, there are some outside the community who will ask whether science can be as deserving of public support as journals such as this repeatedly insist.

One of the prime movers in the discovery of the ring around Neptune appears to have been Professor André Brahic, from the University of Paris and the Observatoire de Paris, who has a long-standing interest in planetary ring systems. Brahic was the first author (among eight) of a manuscript "Occultation detection of a neptunian ring-like arc" which reached the *Nature* office at the end of May this year. The other authors quoted were W.B. Hubbard and F. Vilas from the University of Arizona, who have access to the Inter-American Observatory at Cerro Tololo in Chile, together with L.R. Elicer, a member of the observatory's staff; two colleagues of Brahic's from Paris, B. Sicardy and F. Roques; Patrice Bouchet, a staff member at the European Southern

Observatory (ESO), also in Chile, and two European astronomers, Jean Manfroid from the University of Liege and R. Haefner from the Max Planck Institute of Astrophysics at Munich.

The origins of the dispute among the authors can be traced to the night of 22 July 1984, when there was a near-occultation of Neptune of a bright red star in Sagittarius, then visible from the two Chilean observatories. Brahic and his colleagues in Paris, who seem long to have suspected that Neptune may have a ring system, seem to have taken the initiative in drawing attention to this event. Ironically, Brahic had applied for observing time at ESO on the night in question, but had been given time to look at the rings of Uranus instead. Robbed of this chance, and with the good offices of Bouchet, Brahic seems at second-hand to have persuaded Haefner and Manfroid, the intended occupants of two telescopes at ESO, to turn away from their planned project to look at the occultation by Neptune instead.

In the event, Haefner and Manfroid found that there was indeed a brief second-long interruption of the infrared radiation from the distant star as it crossed an invisible circular orbit roughly 77,000 km nearly 3.0 radii from the centre of Neptune. One curious feature of the observations was that there was no corresponding dip in the radiation intensity as the distant star crossed the same circular orbit at its supposed second interception of it, whence the notion that, if there is a ring around Neptune, it is incomplete.

A similar sequence of events was found at Cerro Tololo, 100 km away in the Andes. Elicer began his observations later in the night, just a few minutes before the expected near-occultation and in some haste, to judge from the circumstance that it is not known what filter had been interposed in one of the two infrared channels. But the results are decisive and essentially the same as those found at the ESO site. Again there was a single interruption of the radiation, lasting about a second but no corresponding dip at the supposed second interception.

It is common-ground that these observations can only mean that there is absorbing material in one part of a supposedly circular orbit about Neptune, but that the material does not extend uniformly around the orbit. Brahic has rightly all along insisted that only coordinated

observations at different sites can lend credibility to such fleeting events. (Clouds, electrical disturbances or passing flocks of birds could be held responsible for a brief interruption of radiation at a single site.) But with a well coordinated pair of observations in hand, Brahic was able to go back to previous attempts to find occulting material around Neptune. The pair of Andean observations last July, from sites separated by 100 km, show that the absorbing material around Neptune must be at least 100 km in extent. The time for which radiation was interrupted suggests an object 15 km across.

So, now there is an interesting and quite novel phenomenon to be explained, a partial planetary ring. If it had been possible to publish the data on which the inference is based, people other than Lissauer would have been stimulated to bend their energies to this interesting problem. But why are the data still under lock and key?

Several things have gone wrong. The ESO observers, Haefner and Manfroid, have been offended by what they consider to have been unfair publicity overwhelming their contributions, a press release from the University of Arizona relayed in semi-popular journals such as *Sky and Telescope*, for example. An article by Brahic in *La Recherche* last June seems to have given particular offence, although all the intended authors of the original *Nature* manuscript are duly listed in the margin. Haefner and Manfroid have published their data in the German-language journal *Stern und Weltraum*.

Much of the difficulty turns on the question of who "owns" the data collected at ESO, the observers who collected it or Brahic who drew attention to the occultation and to whom the data tapes were sent by prior arrangement? And what is to be done with data which, as in this case, are more interesting when combined with other people's than when published on their own?

The saddest feature of this development is that, whoever owns some of the data, there is a sense in which they belong to the scientific community as a whole. The upshot is that an important and even urgent set of data will not now be published quickly, as it should have been. The reputation of astronomers for good sense will have been undermined. Even the reputation of science as a whole will have been damaged — let us hope not irreparably.

John Maddox

Forensic science

DNA fingerprinting in matters of family and crime

from Barbara E. Dodd

IT HAS long been the ambition of the forensic scientist to be able to identify the origin of blood and body-fluid stains with the same degree of certainty as fingerprints. And the ability to settle cases of doubtful paternity with absolute certainty in every case would be equally welcome. The arrival of DNA fingerprinting — particularly as developed by Alec Jeffreys and colleagues at Leicester University^{1,2}, whose latest paper is on page 577 of this issue³ — is a big step towards the forensic scientist's goal in both areas.

As so often happens, the technique has arisen from research that was not aimed specifically at solving practical problems. Jeffreys had been working on the short 'minisatellite' sequence of DNA in an intron of the human myoglobin gene. Using the now familiar methods of Southern blotting and DNA hybridization he found that the myoglobin minisatellite detected other human minisatellites, some of which are highly polymorphic. The common features of these minisatellites have been identified by sequencing, and cloned DNA probes, based on tandem repeats of the core sequences, have been used to detect simultaneously several highly variable genetic loci in the human genome. It is this extreme variability in the pattern of minisatellites detected by the probes,

together with the stable inheritance in the usual mendelian manner of an individual's pattern, that is the basis of DNA fingerprinting.

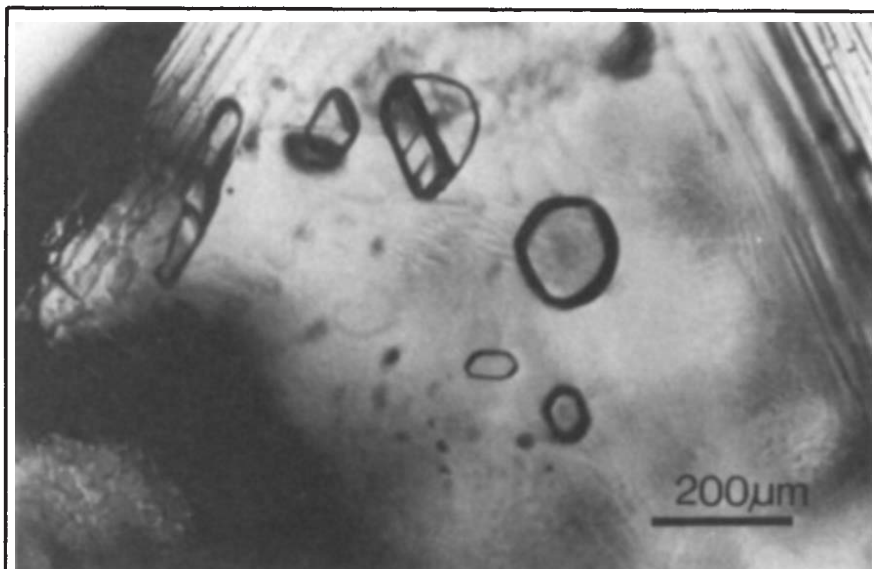
In this issue of *Nature*, Jeffreys with P. Gill and D.J. Werrett of the Home Office Forensic Science Service report promising progress in applying DNA fingerprinting to blood stains, semen stains and hair roots, showing that DNA fingerprints obtained from these sources are always identical to those of the individuals from whom the material originated. Especially rewarding are the results of DNA fingerprints of sperm nuclei and their potential for the identification of rapists. There are conventional genetic markers in semen and semen stains are frequently contaminated with vaginal fluid, so that the origin of any markers present is difficult or well-nigh impossible to trace. Gill *et al.* have managed to isolate sperm nuclei free from vaginal contamination and have obtained a DNA fingerprint that is the same as the donor of the sperm. Thus it should be possible to match a DNA fingerprint of sperm from, for example, a stain on a victim's clothing with that of blood or sperm from a suspect rapist. A chance match is virtually eliminated because its probability is calculated to be of the order of 3×10^{-11} , or even less if more than one

probe is used. The technique, however, has yet to be tested and assessed for application in actual casework. How easily DNA fingerprinting can be assimilated in a routine crime laboratory remains to be established.

Other work is in progress on the feasibility of using DNA polymorphisms in the examination of blood and semen stains. M. Baird and colleagues of Lifecode Corp., New York reported at the 11th International Congress of the Society for Forensic Haematogenetics in Copenhagen last August. They have used probes that recognize two highly polymorphic DNA sequences to show that DNA recovered from blood stains up to three years old can be used for identification purposes and that the DNA fingerprints of sperm recovered from female volunteers after sexual activity, when freed of female cells, is identical to that of the male partner's sperm.

The numerous blood-group polymorphisms that are currently applied to cases of doubtful parentage (usually doubtful paternity) already go far towards excluding every man wrongly named as father of a child. Exclusion of paternity is established by showing the absence in the putative father of one or more blood-group gene products, which, in the child, are seen to be paternally inherited. A range of polymorphic systems are investigated independently of each other by relatively simple procedures in which the gene products are disclosed by agglutination, cytotoxicity tests or some form of electrophoresis. The table shows how a combination of polymorphisms can approach the ability to exclude all false fathers. Moreover, the calculated probability of paternity for unexcluded men, virtually all of whom will be true fathers, is likely to be significantly in favour of their paternity.

Even so, DNA fingerprinting with the probes developed by Jeffreys has the potential to exceed the efficiency of the conventional systems because there are good grounds for anticipating that definitive answers, not only those excluding paternity but also for pinpointing the true father, will be obtained in every case. But it is in resolving problems of family relationships in which putative parents are closely related to each other that DNA fingerprinting has particular advantages over presently used systems. This has already been demonstrated by Jeffreys *et al.*³; the problem was that of a Ghanaian boy who was refused entry into Britain because the authorities were not satisfied that the woman claiming him as her son was in fact his mother. Analysis of a range of systems similar to those in the table showed that the alleged mother and son were almost certainly related (probability of non-relationship 0.01) but the conventional tests were not capable of determining whether the woman was the boy's mother or aunt. DNA fingerprinting established that the mother-son rel-



Unique garnets in diamonds

Enlarged view of a diamond, from the Monastery Mine kimberlite pipe in South Africa, that contains two garnet inclusions (on the right-hand side, with dark shadows surrounding them) and four jadeitic clinopyroxene inclusions. The garnets are the first of natural occurrence to contain pyroxene in solid solution, which indicates an unusually deep origin for the diamonds. This discovery and its implications for mantle structure are described by R.O. Moore and J.J. Gurney on page 553 of this issue.

ationship was correct because the mini-satellites detected by the probes are so hypervariable that the chance of a sister of the alleged mother sharing all the maternal specific bands of the child was 6×10^{-6} .

DNA polymorphisms have also been investigated with respect to US paternity problems, as reported by I. Balazs at the Copenhagen congress. He and his colleagues at Lifecode Corp. have examined DNA polymorphism associated with two genetic loci in more than 100 paternity cases and have shown that their probes produce an exclusion probability similar to HLA (see table). So far their results have not been presented in court. The American Association of Blood Banks committee on parentage testing has studied the implications of paternity testing by DNA and has issued guidelines. These include the need to validate DNA probes by extensive family and population testing before they are used in parentage testing; the estimation of gene frequency and mutation rate for the genes being detected; the inclusion of duplicate gels and blots; and, at least for the present, parallel testing with accepted systems.

Although DNA fingerprinting as developed by the Jeffreys team requires but a single series of manipulations to disclose a richer variety of polymorphisms than shown in the table, the process is very labour-intensive and needs both meticulous expertise and much experience in the reading and interpretation of the bands that appear in the final autoradiographs. And since the outcome in human terms is likely to be far reaching and since no confirmatory tests using other independent systems are possible, it is surely essential to duplicate each test. A two-tier procedure may develop, beginning with a range of conventional systems and proceeding to DNA fingerprinting in selected cases.

One can foresee that through the energy of molecular biologists, probes suit-

Change of exclusion of non-fathers

System	Chance (%)
Red cell antigens	
MNSs	32.1
Rh	28.0
Kidd	19.0
Duffy	18.0
ABO	17.6
Kell	3.3
Lutheran	3.3
Serum proteins	
Gc	24.7
Hp	17.5
Glm	6.5
Km	6.0
Red cell enzymes	
Phosphoglucosmutase	25.3
Erythrocyte acid phosphatase	21.0
Glutamate - pyruvate transaminase	19.0
Glyoxalase	18.4
Esterase D	9.0
Adenylate kinase	4.5
Adenosine deaminase	4.5
HLA	94.0
Total for all systems combined	99.7

able for appropriation by forensic scientists will proliferate while techniques for DNA fingerprinting may become simpler. Excitement concerning the application of DNA fingerprinting to forensic science has to be tempered by the realization that much assessment remains to be done. If this proceeds satisfactorily it will be difficult to disagree with the prediction of Gill *et al.* that DNA fingerprinting will revolutionize forensic biology. □

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mass of the asteroid zone would have remained reasonably constant throughout most of the life of the Solar System, the main loss having occurred as a result of the primordial process that stirred up the zone — possibly perturbations by a proto-Jupiter. This stirring changed the low interparticle velocities at which accretion could take place into much higher impact velocities which resulted simply in collisional fragmentation.

One way of discriminating between these two possibilities is to look at the collisional evolution of the present asteroid belt and to wind this back to see if one gets a reasonable initial population or not. If the population is large the first process is sufficient. The second process would indicate that the asteroid zone has had a low mass ever since the growth of proto-Jupiter. This has significant implications for the degree to which asteroidal spins have been modified by collisions and for the amount of fragmentation that asteroids have suffered.

Consider the observational constraints. Davis *et al.*¹ note that Vesta (the fourth largest asteroid, diameter 480 km) has a largely intact basaltic crust, indicating that it has not been shattered during the pre-primordial period and that the number of bodies capable of breaking up Vesta must be low. A significant number of asteroids are members of families having similar orbits and having been produced by the disruption of a single parent, generally thought to have a diameter about 200 to 300 km^{1,2}. The fact that the families persist indicates that collisions are rare.

A third, less strict, constraint comes from collision modelling¹. The frequency of collisions at 5 km s⁻¹ between asteroids of, say, 100-km and 500-km diameter is quite well known but the outcome of the collisions is not. In laboratory studies, collision energies reach around 10¹¹ ergs, which is less than 10⁻²¹ of the kinetic energy of a body with a 100-km diameter.

Farinella *et al.*² turn to the rotational properties of asteroids as the fourth constraint. About one third of the asteroids in the 200–300-km-diameter range seem to have elongated shape, indicated by large light-curve amplitudes and rapid spins. If this was induced by collisions there must have been about five times as many asteroids larger than 65 km in the initial (post-primordial) population than there are now. A similar conclusion is reached by Davis and colleagues³.

The history of the asteroid zone is still somewhat vague. Greenberg *et al.*⁴ found that it would take about 10⁷–10⁸ years to accrete Ceres if there was initially a total rock mass of about the mass of the Earth, all in the form of 1-km bodies. Then something must intervene to terminate the accretion. Maybe it was orbital stirring by proto-Jupiter. If so, Jupiter has to accrete very quickly, more quickly than the asteroids, and one is still left with the problem that the stirring process itself takes about

Solar System

Missing mass in the asteroid zone

from David W. Hughes

LIKE the Cassini division in Saturn's rings, there is a division in the Solar System, at the asteroid zone. Both these divisions indicate regions of high mass depletion. At present, the asteroid belt between the orbits of Mars and Jupiter contains an observed¹ mass of about 5×10^{24} g, about one thousandth the mass of the Earth. If the distribution of mass in the proto-planetary solar nebula were reasonably uniform there would have originally been sufficient rocky material in that zone to form a planet or planetary core of at least the same mass as the Earth. What has happened to this mass? Farinella, Paolicchi & Zappalà² suggest two alternatives —

either there has been continued gradual loss of mass or the main mass was lost primordially and little has been lost since.

The gradual process would result from asteroids breaking up as they collide together at a mean speed of around 5 km s⁻¹. Most of the debris has speeds well above the escape velocities of the two colliding asteroids and so move off into the zone. The Poynting–Robertson effect, that is the symmetrical re-radiation of absorbed unidirectional solar radiation, retards the dust, causing it to spiral in towards the Sun. Over the age of the Solar System this has depleted the asteroid population. In the second process, the

10⁶ years. The orchestration of this process has to be finely tuned. If Jupiter formed too early, premature stirring would have prevented the accretion of large Ceres-type asteroids. If Jupiter formed too late, accretion in the asteroid belt would have continued for too long and there would now be a planet there. It was probably close run — if Ceres were about twice its present size, it would accrete even at impact velocities of 5 km s⁻¹.

Another suggestion⁷ is that accretion stopped and the asteroid velocities were increased by secular resonances sweeping through the zone. These were caused by mass loss from the Sun, this mass being dissipated in the solar nebula during the Sun's T-Tauri phase. Unfortunately, for this to work, the T-Tauri phase would have to be exceedingly active and to have lasted for about 10⁷ years.

Whichever velocity-increasing process we choose, it still has to explain how the original mass of the zone (one Earth mass) was reduced to the post-primordial mass, which was five times greater than it is now. Explaining the present state of the asteroids and their origin is still a major task in Solar System science. □

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Cell biology

Traffic control and structural proteins in the eukaryotic nucleus

from Larry Gerace

ALTHOUGH RNA and proteins are exchanged continuously between the nucleus and cytoplasm in most eukaryotic cells, these two compartments each maintain a distinctive macromolecular composition. The nuclear envelope, the double membrane structure that forms the boundary of the nucleus, is thought to be a major regulator of these differences. Results discussed at a recent meeting* and in a number of publications augur a new period of progress for understanding the regulation of nucleocytoplasmic traffic, as well as the molecular organization of the nuclear envelope and lamina.

Protein import

Molecules are translocated across the nuclear envelope through proteinaceous channels called nuclear pore complexes (see the figure), which are probably the major regulatory sites for nucleocytoplasmic transport. The pore complex has an elaborate architecture that has been characterized by three-dimensional reconstruction from electron micrographs¹. Whereas it contains a mass that may exceed 25–50 × 10⁶ daltons, almost nothing is known about its polypeptide composition. From microinjection studies, it is known that these proteins that are much more concentrated in the nucleus than the cytoplasm ('karyophilic' proteins) possess information in their mature polypeptide structure that is responsible for their accumulation in the nucleus^{2,3}. Since the pore complex seems to contain an aqueous channel with a functional diameter of about 10 nm (ref. 4 and R. Peters, Max

Planck Institute for Biophysics, Frankfurt), the rapid *in vivo* uptake of small (relative molecular mass, M_r , less than about 20,000–40,000) globular karyophilic proteins by the nucleus can, in theory, be explained by passive diffusion through the pore complex followed by intranuclear binding. The size of larger macromolecules, including many proteins and RNAs, would severely restrict their passive diffusion, and their translocation across the nuclear envelope may require a mediated-transport mechanism⁵.

Considerable effort is now being focused on identifying the signals that direct some proteins into the nucleus. A karyophilic protein that has proved to be a good model for studying mediated transport is nucleoplasmin, a 165 M_r , pentameric molecule. Using limited proteolytic digestion, it has been determined⁶ that the nucleoplasmin monomer has a discrete 10 M_r , 'tail' that contains the information mediating the selective nuclear uptake of this protein (R. Laskey, Cambridge University). In a striking experiment⁶, injection of nucleoplasmin-coated gold particles into the cytoplasm of frog oocytes has been shown to result in rapid nuclear uptake through the pore complex of particles with diameters as large as 20 nm (C. Feldherr, University of Florida, Gainesville). This suggests that the signal in nucleoplasmin causes the pore complex to become a 'gated' structure.

Recombinant DNA procedures have precisely defined the nuclear localization signal in the simian virus 40 T antigen^{7,8}, another large (94 M_r) karyophilic protein. A short sequence of the T antigen molecule (amino acids 126–132) is both suffi-

cient and necessary for the nuclear localization of this protein, and if this sequence is experimentally attached to large proteins that are normally cytoplasmic, it can retarget them to the nucleus (A. Smith, Integrated Genetics). Because a similar sequence is found in other viral nuclear proteins, it may be a prototype for one class of nuclear localization signal. However, as shown for the yeast MAT $\alpha 2$ protein, two distinct sequences on different regions of the same polypeptide can each be sufficient for nuclear uptake (ref. 9 and M. Hall, University of California, San Francisco).

The key issue to be addressed at this point is the nature of the cellular structures that recognize the protein signals for nuclear accumulation. Are they part of the nuclear pore complex or are they binding sites (chromatin or otherwise) within the nucleus? When the T antigen is expressed in cells of the alga *Acetabularia*, it also becomes concentrated in the nucleus (G. Neuhaus, Max Planck Institute for Cell Biology, Ladenburg), suggesting strong phylogenetic conservation of the nuclear uptake apparatus for this protein.

An interesting mechanism that is used by cells to regulate import of nuclear proteins has come to light from developmental studies with *Xenopus*. The proteins that are complexed with U2 RNA to form a small nuclear ribonucleoprotein particle (snRNP) are stockpiled in the cytoplasm of *Xenopus* oocytes in the absence of associated RNA, and only accumulate in the nuclei of embryos after transcription of the genes for U2 RNA starts at the mid-blastula stage of development¹⁰. Microinjection of U2 RNA into oocytes induces assembly of these U2-binding proteins into snRNP, which then migrate into the nucleus.

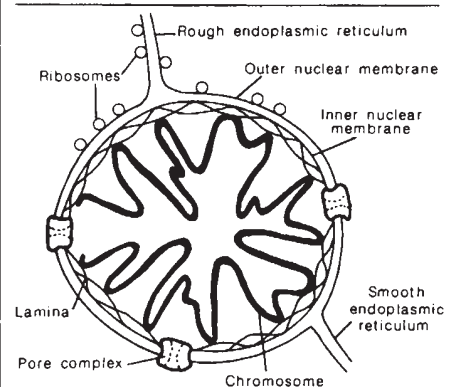


Diagram of the major structural components of the nuclear envelope, consisting of inner and outer nuclear membranes that are joined at the pore complexes, proteinaceous supramolecular structures that provide channels for molecular movement between the nucleus and cytoplasm. The outer nuclear membrane frequently has ribosomes attached and is structurally continuous with the rough and smooth endoplasmic reticulum. The nuclear lamina, a filamentous protein meshwork lining the nucleoplasmic surface of the nuclear envelope, probably provides an anchoring site at the nuclear periphery for interphase chromosomes.

*The Workshop on Nucleocytoplasmic Transport was held in Heidelberg, FRG, 26–28 September, 1985.

Mutant forms of U2 RNA that are not assembled into normal snRNP do not cause nuclear accumulation of the same proteins (ref. 11 and I. Mattaj, University of Basel). This indicates that karyophilic 'domains' are generated by RNP assembly, perhaps by conformational change. A variation on this mechanism may explain how certain proteins that are initially present in the nucleus of *Xenopus* oocytes become cytoplasmic during early cleavage stages of embryogenesis, and re-enter nuclei only at precisely defined periods later in development¹².

Encouraging progress was reported on the development of cell-free systems to study nuclear uptake of large proteins. Starting with isolated DNA or sperm chromatin and extracts of *Xenopus* eggs, several groups have assembled nuclei *in vitro* based on previous procedures^{13,14} which can accumulate nucleoplasmin (T. Burglin, University of Basel), nucleoplasmin-coated gold particles (R. Laskey) or endogenous karyophilic proteins of oocyte nuclei (C. Dreyer, Max Planck Institute for Developmental Biology Tübingen). Uptake of nucleoplasmin requires ATP both as *in vivo* as well as *in vitro* (T. Burglin), which is consistent with the operation of an active transport mechanism. Cell-free systems should offer a powerful approach for studying nucleocytoplasmic transport, particularly when immunological probes to the nuclear pore complex become available.

Lamina revealed

The nuclear lamina is a supramolecular assembly that lines the inner surface of the nuclear envelope and may both regulate nuclear envelope structure and anchor interphase chromosomes at the nuclear periphery¹⁵. In cells of several higher eukaryotes, the lamina consists mainly of a small number of related 'lamin' polypeptides. At least in *Xenopus*, these proteins are coded by a developmentally regulated gene family¹⁶, and the major lamins found in germ cells are distinct from the lamins of many somatic cells (G. Krohne, German Cancer Research Centre, Heidelberg).

Three discrete 60–70 M_r lamins (A, B and C) are present in typical mammalian somatic cells. The first amino-acid sequences of lamin A (G. Blobel, Rockefeller University) and, independently, of both lamins A and C¹⁷ have recently been deduced from human cDNA clones. Interestingly, a region of about 300 amino acids near the amino terminus of the lamins possesses striking sequence homology (greater than 80 per cent in certain regions) with the canonical 300-amino-acid, coiled-coil, alpha-helical rod domain that characterizes all intermediate filament proteins. A large domain with little alpha helix is predicted to occur at the carboxy terminus of the lamins. Electron microscopy in my laboratory (Cohn, J. & Gerace, L. in preparation)

shows that the three lamins from rat liver (which are apparently dimers) each contains a globular head attached to an approximately 50 nm rod-like tail. The latter bears close resemblance to the alpha-helical rod of intermediate filament subunits. Together with a recent demonstration that the lamina of *Xenopus* oocytes consists of filaments with a diameter of 8–10 nm arranged in a regular near-tetragonal lattice (Buhle, L. & Aeby, U. in preparation), these data indicate that the lamins constitute a hitherto unrecognized class of intermediate filament proteins.

Unlike many intermediate filament proteins of the cytoplasm, the lamins undergo total structural reorganization during mitosis in mammalian cells, when they are reversibly depolymerized to a 4–5S form by a process that may involve lamin hyperphosphorylation^{18,19}. An attractive possibility is that reversible disassembly of the lamina regulates the disassembly and reconstruction of the nuclear envelope that occurs during mitosis in higher eukaryotes. In support of this notion, I reported that removal (by immunoadsorption) of the disassembled lamins from a cell-free nuclear envelope assembly system results in strong inhibition of subsequent nuclear envelope formation. Mitotic-like disassembly of isolated nuclei can be induced by extracts from *Xenopus* eggs arrested in metaphase^{19,20} (J. Newport, University of California, San Diego) suggesting that purification of lamina disassembly factors is feasible. Thus analysis of lamina disassembly may ultimately shed light on the biochemical regulation of mitotic prophase which, in one intriguing scenario²¹, may involve a phosphorylation cascade.

Only the lamina of higher eukaryotes has been characterized biochemically, but

it is also present in at least some lower eukaryotic organisms; indeed, it was first described structurally in *Amoeba proteus*²². It is conceivable that the lamina emerged early during eukaryotic evolution, perhaps to carry out the functions of anchoring chromatin to the nuclear envelope and regulating nuclear envelope growth. The lamins may therefore represent an ancient class of intermediate filament proteins. Although the last in the current line of intermediate filament proteins to be recognized, they are certainly not the least interesting. □

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Palaeoclimatology

Cycles in the Precambrian

from A. Barrie Pittock

CLAIMS for the influence of sunspot cycles on the weather are highly controversial^{1–3}, so clear evidence for such an influence is an exciting prospect. Our best source of information is a series of periglacial varves in deposits from lakes in Australia. These varves are layers of silt laid down annually in a lake below the outlet of a glacier. They were first documented in 1981 by Williams⁴ from layering observed in a short section of exposed rock at Pichi Richi Pass in the Flinders Ranges. On page 523 of this issue G.E. Williams and C.P. Sonett present data from several drill cores giving a continuous stratigraphic sequence spanning some 19,000 'annual' layers⁵ which provides overwhelming statistical evidence that such cyclical activity really existed over a long period in the geological past.

Ever since sunspot cycles were first documented by Heinrich Schwabe in 1844, the possibility that they influence weather and climate has fascinated amateur and professional meteorologists^{6–9}. Claims for solar cycles in meteorological data have usually not been strong enough to overcome the variance in the data or to be obvious in visual inspection of time-series plots — the exceptions have in general been found to be spurious¹. Numerous small and marginally significant cycles have been proposed as the result of the statistical massaging of large quantities of meteorological data but most of these have again proved to be either spurious or at best controversial. Indeed, the noted Russian meteorologist A.S. Monin has characterized the field as one that is remarkable for "successful experi-

ments in autosuggestion^{10,11}.

The cyclical appearance of the varve sequence from Pichi Richi Pass is neither weak nor statistically marginal. Rather, it is visually obvious, and indeed dominates the total variance in the time series. The analysis by Williams and Sonett shows an impressive series of periodicities, including a basic cycle of approximately 12 'years', with alternating thick and thin cycles, and longer-term rhythmic fluctuations in the period every 13 cycles. Spectral analysis reveals a harmonic series with a fundamental period consisting of about 26.1 of the 12-year cycles and higher harmonics at 13.1, 8.8, 6.6 and 5.3 cycles.

Although the evidence for cycles in the varve sequence is overwhelming, there are problems concerning any interpretation of its physical significance. It is difficult to imagine any other explanation for the layered deposits than that of an annual meltwater cycle but the authors put 'years' in quotation marks, presumably to indicate that they have nothing more than circumstantial evidence for the annual origin of the layers. Similarly, the identification of the cyclical appearance with cycles of solar activity is largely circumstantial; we have no direct evidence for the cyclic behaviour of solar activity some 680 million years ago, so correlation of varve cycles with solar activity can only be hypothetical. Indeed, the authors imply that in future papers they will use information derived from the varve sequence to help with modelling solar behaviour — a procedure that would introduce a strong element of circularity into the argument.

Williams and Sonett interpret the varve sequence as indicating that increases in solar activity caused corresponding increases in climatic temperature, resulting

in greater annual meltwater discharge and the deposition of thicker varves. This is not consistent with a direct relationship between solar irradiance and surface temperature, since satellite measurements indicate that at times of increased solar activity, as indicated by sunspots, there is a slight decrease in solar irradiance¹². Of course, temperature at a particular locality on the Earth may be influenced by topographic or other local effects and so may not vary directly with fluctuations in solar irradiance.

Perhaps the biggest question posed by the varve sequence is why cycles should be so obvious in rocks about 680 million years old, when they are not strongly in evidence in the modern record. Williams and Sonett cite the existence of "a relatively weak solar signal" in recent varves from Skilak Lake in Alaska¹² but if this is the best evidence for the influence of solar cycles that can be found in recent times it leaves much to be explained. □

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100 years ago

THE Government of Tasmania are making arrangements upon a large scale for naturalising lobsters, crabs, turbot, brill, and other European fishes in the waters of that country. The various consignments will be shipped at Plymouth, and transported through the medium of the steamship companies trading between London and Hobart. An exhaustive report has been published by the Government of Tasmania, setting forth the objects in view, and giving suggestions for carrying them into effect. The report adds that while the achievement of the acclimatisation of European fishes would lay the foundation of new and very valuable fishing industries in Tasmania, it might also prove a highly remunerative commercial enterprise to the shipping firms under whose auspices the operations will be conducted. Applications have been made for the supplies of fish, which have been satisfactorily responded to. Special tanks are being prepared in order to provide for the necessities of the fish *en route*.

From *Nature* 33 137, 10 December, 1885.

are associated with the activation of *c-myc* disrupt its regulation? One possibility is that they place *c-myc* under the control of a constitutively active gene enhancer so that it is no longer responsive to the normal cellular controls. This is consistent with the finding that the normal *c-myc* allele in tumour cells containing one activated *c-myc* gene is almost always silent¹⁰.

An alternative possibility is based on highly suggestive evidence implicating the 5' exon of the cellular gene in its regulation. This exon, which is not translated, is missing from the *v-myc* oncogene that is found in some tumour viruses and is very often lost from the cellular gene as a result of the rearrangements in tumour cells. This has led to the proposal¹¹ that *c-myc* is under negative regulation acting on the 5' end of the gene or its flanking sequences through the binding of a repressor protein. The experiments of Adams *et al.*, however, now show that the activation of *c-myc* is almost certainly independent of the presence or absence of 5' sequences; while the most recent evidence on the normal regulation of the gene suggests that the 5' exon may be important not in the control of *c-myc* transcription but in its post-transcriptional regulation.

The evidence that *c-myc* is normally transcriptionally regulated comes from measurements of *c-myc* mRNA. The induction of cell proliferation by mitogens dramatically increases *c-myc* mRNA levels¹, while the cessation of cell proliferation that accompanies differentiation leads to an equally dramatic reduction¹¹. These changes were initially assumed to be in the transcription rate of the gene. However, although a rapid induction of *c-myc* transcription is found in serum-stimulated fibroblasts¹², the 3–4 fold increase is not sufficient to account for the 20–40 fold increase in mRNA level. Furthermore, after a transient increase, the rate of transcription returns to the pre-induction level while the cytoplasmic RNA level remains high.

Oncogenes

Regulation and activation of *c-myc*

from Michael D. Cole

THE regulation of the *c-myc* oncogene and its role in the cancerous transformation of cells have been among the most widely studied and controversial subjects in the study of the molecular basis of cancer. This interest has been stimulated largely by the discovery that *c-myc* genes that have been activated by a variety of mechanisms (reviewed in ref. 1) are characteristic of a very wide range of tumours. Since the expression of *c-myc* is also characteristic of normal proliferating cells, however², it has become clear that in order to understand the nature of the abnormal activation of *c-myc* in tumour cells it is essential to understand how it is normally regulated. The paper by Adams *et al.* on page 533 of this issue³ provides important insight into the mechanism of abnormal *c-myc* activation and a number of recent studies have revealed an unexpected

complexity in the normal regulation of *c-myc* expression.

Two of the possible mechanisms of *c-myc* activation can now be dismissed. First, it is clear that the levels of *c-myc* messenger RNA are no higher in normal proliferating cells than in tumour cells, so that activation is not caused by overexpression⁴. Second, the expression of *c-myc* is constant throughout the cell cycle in normal cells⁵, so earlier suggestions that the gene is cell-cycle regulated in normal but not in tumour cells are not borne out. This has led to the alternative suggestion that activation involves the loss or disruption of control elements that enable the normal gene to be switched off when the cell enters the quiescent, non-proliferating state. The question then becomes, how do the chromosomal rearrangements and retroviral insertions that

More recently it has been established that the major cause of rapid changes in *c-myc* mRNA levels is a modification in post-transcriptional RNA stability. The levels of *c-myc* mRNA decrease rapidly both in lymphoblasts treated with interferon¹³ and in teratocarcinoma cells induced to differentiate with chemicals¹⁴. This occurs without any change in the rate of transcription, possibly because of a substantial decrease in the already short half-life of *c-myc* mRNA¹⁵. An increase in mRNA levels without a change in transcription rate is also seen in secondary lung fibroblasts¹⁶. In the light of recent evidence for a role for 5'-untranslated sequences in posttranscriptional regulation, it seems possible that the untranslated first exon of *c-myc* is the major target for destabilization or transport of the transcript. Post-transcriptional regulation at the level of RNA turnover therefore seems to account for much of the modulation of *c-myc* expression. These findings, however, still do not account for *c-myc* activation by the many proviral insertions and translocations that do not affect *c-myc* RNA structure.

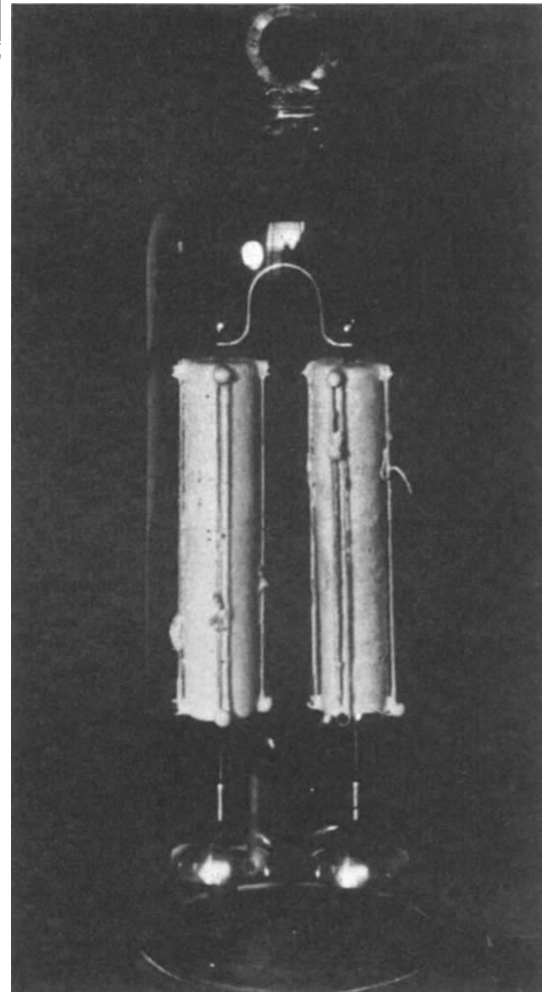
Adams *et al.*³ have addressed this problem using transgenic mice carrying different configurations of normal *c-myc* or *c-myc* genes linked to enhancer sequences from immunoglobulin genes. The results are quite striking and provide an elegant demonstration of the transforming potential of activated *c-myc* genes. Injection of *c-myc* genes joined to either the immunoglobulin heavy-chain or light-chain enhancers led to the induction of multifocal lymphomas in 90 per cent of the mice, while intact or truncated normal *c-myc* genes (without enhancers) produced no neoplastic disease. These findings are analogous to those of Stewart *et al.*¹⁷ in which a *c-myc* gene linked to a 'long terminal repeat' sequence of the mouse mammary tumour virus was shown to potentiate mammary carcinomas. In both studies, a tissue-specific control element apparently induces high levels of *c-myc* expression leading to a heritable susceptibility to tumours. Adams *et al.* clearly demonstrate that enhancement or transcriptional activation of *c-myc* is necessary to induce its oncogenic potential, while neither the alteration of its chromosomal position nor its truncation are sufficient for activation. However, this study does not provide an explanation of the mechanism of activation of *c-myc* transcription in the majority of Burkitt lymphomas and mouse plasmacytomas, where the immunoglobulin enhancer and other sequences that are known to control immunoglobulin gene expression are not present on the chromosome containing the *c-myc* coding exons and thus cannot influence the expression of the translocated gene. Presumably there is some unidentified transcription-activating sequence or change in chromatin configuration in these other cases.

By analysing not only *c-myc* expression

but also immunoglobulin rearrangements, Adams *et al.* obtained important additional information. First, the majority of the tumours (which were of both pre-B and B-cell stages) had a distinct set of rearranged immunoglobulin genes, which indicates that the tumours were clonal. Because the presence of the immunoglobulin-enhancer-linked *c-myc* gene in the germ line should lead to high levels of *c-myc* expression in all lymphocytes, this result suggests that a second event may be required to generate a fully transformed cell.

Furthermore, Adams *et al.* find that, whereas the enhancer-linked *c-myc* gene is expressed in the tumours, the endogenous *c-myc* gene is not. This makes the induced tumours analogous to natural tumours with transcriptionally activated *c-myc* genes, such as plasmacytomas¹⁸ and Burkitt lymphomas¹⁹. In the light of the transcriptional and post-transcriptional control of *c-myc* expression discussed above, it will be very interesting to learn at what level the endogenous gene is down regulated. Adams *et al.* argue that the lack of endogenous *c-myc* expression is consistent with an autoregulation control model, in which expression of the activated allele suppresses the expression of the germ-line gene. However, as the authors note, they cannot exclude the possibility that the transformed B cells in their study arose from a cell in which the endogenous *c-myc* gene is not normally expressed. Furthermore, autoregulation is not consistent with observations of expression of the endogenous *c-myc* gene in some of the mammary carcinomas induced by *c-myc* linked to the 'long terminal repeat' sequences of mouse mammary tumour virus¹⁸, nor with the effects of introducing a constitutively expressed *c-myc* gene into fibroblast lines that are already expressing *c-myc*; in this case, again, the introduced gene does not affect the expression or regulation of the

Long-running experiments (III)



A dry pile has driven this electric bell since sometime before 1840 when it was re-set up in Oxford University's Clarendon laboratory. The clapper draws about 1 nA as it oscillates between the terminals at a speed which declines with humidity. Occasionally humidity stops the movement, which starts again spontaneously. It is not known what the piles are made of. More details in *European Journal of Physics* 5, 193; 1984, which invites "accounts of other experiments that call for patience on the part of the recorder".

endogenous gene¹⁸.

Is it possible that the 'second event' that seems to be required for transformation normally turns off the *c-myc* gene in a manner analogous to that seen in terminal differentiation, and that DNA rearrangements or proviral enhancers prevent this down regulation? Despite the progress that has been made in understanding the regulation of this complex and interesting oncogene, clearly many challenging questions are still unanswered. □

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Mathematics

Classical continued fractals

from Ian Stewart

CONTINUED fractions are classical: they provide 'best possible' rational approximations to real numbers, expressed in the form $\alpha = 1/(a+1/(b+1/(c+1/...)))$, usually written as $[a, b, c, \dots]$ for simplicity. Their main use is in number theory. Fractals are of much more modern vintage. They are geometrical objects with structure on all scales. The name was coined by their inventor, Benoit Mandelbrot, to reflect their 'infinitely crinkled' shape. They are applied to irregular behaviour and form in nature.

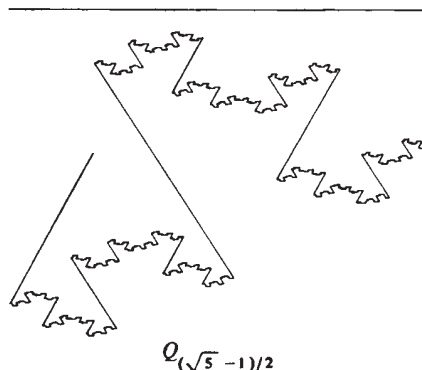
Can two such disparate ideas usefully be combined? Indeed they can. Jenny Harrison of the University of California at Berkeley has announced progress on a baffling problem known as the Seifert conjecture based on what she calls continued fractals. The proof is sketched in *Bulletin of the American Mathematical Society* 13, 147; 1985; full details will appear elsewhere.

The celebrated 'hairy ball' theorem states that it is impossible to comb a hairy ball smooth. More precisely, any smooth vector field on a two-dimensional sphere must have a fixed point. In consequence, any smooth dynamical system whose state space is topologically a two-dimensional sphere must have at least one steady state. On the other hand, a hairy torus can easily be combed smooth, so dynamics on a torus can have no steady states at all. Such results have far-reaching consequences: for example they justify the choice of tori, rather than spheres, for magnetic bottles in experimental fusion reactors.

But what if the state space is a three-dimensional sphere? First we must explain how such an object can be visualized. The circle, a one-dimensional sphere, can be obtained from a line by adding a 'point at infinity' which is considered as lying at both ends, so that the line plus this point curls up into a circle. Similarly the 2-sphere can be thought of as a plane, plus a single point at infinity, lying at both ends of every line in the plane. The 'horizon circle' of the plane is squashed down to this single point; and much as a bag can be closed by tightening a loop around the top, the plane curls up to give a sphere. For a 3-sphere, just add a single point at infinity to three-dimensional space. To

visualize a smooth combing of the 3-sphere, we imagine a family of smooth curves through 3-space, taking care that it behaves suitably 'near infinity'.

It is not hard to find ways to comb a hairy 3-sphere smoothly, with no fixed points. Other special features tend to appear, however, notably places where the hairs form a closed loop. Is there a way to comb a 3-sphere that has no fixed points and no loops? In 1950, Herbert Seifert conjectured that there is not or, more precisely, that every smooth vector field on



Example of a continued fractal (redrawn from Harrison, J. *Bulletin of the American Mathematical Society* 13, 147; 1985).

the 3-sphere has either a fixed point or a closed integral curve. At first, very little progress was made on this fundamental problem, because nobody knew where to start.

To describe the known results we must address the question of how smooth is 'smooth'? The degree of smoothness of a curve is measured by how many times it can be differentiated. To qualify as 'smooth' it must certainly have a tangent everywhere (be once differentiable). If the position of that tangent also varies smoothly, the curve is twice differentiable, and so on. The smoothest curves are infinitely differentiable. A fancier definition lets the degree r of differentiability be fractional, indeed real, as well as integral.

In 1971, Paul Schweizer (*Annals Mathematica* 100, 386; 1971) showed that the Seifert conjecture is false in the once-differentiable case. So for a degree of differentiability r somewhere between 1 and ∞ , there is a switch from false to true (unless, as may be the case, it is always false). The question is, where? In her thesis under Colin Rourke at Warwick University, Harrison showed that the switch is at a value $r > 2$. Her new work, which uses similar methods, improves this to $r \approx 3$, pushing current techniques somewhere near their limit.

The starting point of her method is a

deceptively simple example of discrete dynamics. Take a circle, select a real number α between 0 and 1, and define a transformation T of the circle by rotating it through the angle $2\pi\alpha$. Then, under iteration of this transformation, the behaviour of points is highly sensitive to the degree of irrationality of α . Specifically, suppose α has continued fraction $[a, b, c, \dots]$, then the smaller that the entries $a, b, c, \dots$ are, the more irrational is α .

When the rotation α is rational, every point on the circle is periodic under iteration of T . That is, if T is performed sufficiently often, every point ends up exactly where it started. If α becomes irrational, this periodicity starts to break up. For highly irrational α , such as the golden number $[1, 1, 1, 1, \dots]$, the iterates under T are evenly spread over the circle. For 'almost rational' numbers, like the Liouville number $[1, 2, 2^2, 3^3, \dots]$, the points tend to clump together during long sequences of iteration. Thus there is a strong link between the dynamics of T and the continued fraction of α .

There are well known techniques for constructing flows on the 3-sphere from transformations such as T , and the central question is to determine how the degree of differentiability of the resulting flow depends on the degree of irrationality of α . The falsity of the Seifert conjecture unless $r > 2$ comes from taking $\alpha = [4, 4, 4, \dots] = \sqrt{5} - 2$. Can other choices of α lead to smoother flows?

It is here that continued fractals enter. Harrison describes a geometric way to visualize the continued fraction of α , by 'unfolding' the sequence of iterates of a point under the rotation T . This leads to a curve whose geometric structure determines arithmetical properties of the continued fraction. The curve is a fractal, and its geometric fine structure corresponds to delicate arithmetical properties, making it possible to visualize the way in which α is approximated by rationals, and to apply geometric reasoning. The improved value $r \geq 3$ arises by taking $\alpha = [N, N, N, \dots]$ for large and even values of N , and by exploiting the interplay between dynamics, geometry and arithmetic. It is a vivid display of the unity of mathematics.

Meanwhile, the general Seifert conjecture remains as enigmatic as ever. Conceivably it is false even in the infinitely differentiable case. There seems little chance that current techniques can lead to a general counter-example. Harrison's methods break down for r larger than 3, leading her to suggest that it is at $r = 3$ where the conjecture turns into a theorem. However, no proofs exist for any r, ∞ included; other considerations suggest that a proof (if indeed the conjecture is ever true) must overcome formidable technical obstacles. We may have a long wait for the complete answer. □

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Errata

Throughout A.M.C. Sengor's article "East Asian tectonic collage" (7 November, p.16) the term "Indosinian" should replace "Indonesian", which was inadvertently substituted during editing.

In Michael Brown's article "Tectonics of metamorphism" (28 November, p.314), the wrong photomicrograph was used to illustrate Fig.2.

Another reason for saving Sakharov

SIR—I believe it is crucial in our attempts to rescue Andrei Sakharov to re-emphasize Sakharov's scientific legacy to the scientific community at large and to Soviet officials in particular. Studying recently an early paper on gravitation theory by Sakharov¹, I realized that this work not only provides a new way of looking at gravity, but also gives us a new weapon in the battle for Sakharov's life. The paper, which describes the possible connection between gravitation and vacuum fluctuations, has been referred to in a number of major textbooks²⁻⁴. My own investigations have given me an even higher opinion of this seminal paper.

I have been studying the influence of a suggested inhomogeneity in zero-point vacuum fluctuations on free particle motion and have come to the conclusion^{5,6} that in the general case the particle world-lines become geodesics of a Riemannian geometry. So I was able to appreciate Sakharov's main suggestion of a direct connection between the vacuum fluctuations and gravity (that is, Riemannian geometry of spacetime). In the appropriate sense, Sakharov obtains the Hilbert Lagrangian of general relativity. As a result the Einstein equations acquire the meaning of connection between an inhomogeneity in the vacuum fluctuations and the distribution of matter. Some formal difficulties in the development of Sakharov's concept are removed by the recently derived covariant description of gravitational energy.

I believe that Sakharov's concept may resolve the 60-year confrontation between quantum field theory approaches to gravity and Einstein's general relativity, after hundreds of futile attempts to bridge this gap. Once some formal difficulties are removed, I anticipate the development of the physics of vacuum fluctuations as a fundamental branch of science with many applications. Hence I believe that historically Sakharov's name will be among the most illustrious names in science. Like Evariste Galois, another genius of the highest originality, Sakharov chose the way dictated by his moral self-obligation and this deprived him of the opportunity to advance many of his ideas. Like Galois, he gave only a short synopsis of his concept. I believe that the concept will steadily increase in importance to science, as did Galois' contribution.

Hence I perceive a new starting point in our efforts to save Sakharov's life. His humanitarian activities have been completely rejected by Soviet authorities as part of the dangerous "bourgeois ideology" with which they claim no conciliation is possible. They do not think that Sakharov's role in the creation of Soviet nuclear weapons is worthy of reward or respect in the West. He is not considered as a head of

some scientific school inside the Soviet Union or abroad. So, to Soviet authorities, Sakharov is nothing but a Western tool of the cold war. They assume that he will be forgotten in the West as soon as he is out of sight. Hence, from their point of view, Sakharov may safely be ignored. Perhaps the new Soviet leaders will rectify this situation when they realize that his scientific legacy will not be forgotten by history and that Sakharov's persecution will be recalled in every physics textbook.

It is important to make it clear to the Soviet authorities (and perhaps to some of us also) that Sakharov is likely to be seen as the founder of a new fundamental branch of physics which may find wide application. I believe that the best way to advance this understanding is the rapid development of Sakharov's concept and its broad explanation. My belief is that the investment of money and intellectual resources in Sakharov's concept are completely justified by the expected scientific results. But there is an additional justification. The rescue of Sakharov is our ticket of admission to the human race.

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Ocean thermal response in man-made climatic change

SIR—John Woods has raised¹ important issues concerning the dynamic response of ocean circulation to anthropogenically imposed changes in atmospheric CO₂ level. The full implications of the physical characteristics he presented have potentially serious environmental policy consequences beyond those discussed in his paper. The delay in ocean-atmospheric temperature equilibration due to the ocean's thermal mass following the introduction of man-made infrared absorbing trace gases into the atmosphere implies a slower rise in global climatic warming than given by calculations that omit oceanic effects. The time it would take to reach the conventional measure of a significantly hazardous environmental condition (a global temperature rise of about 3°C, which corresponds to doubling of atmospheric CO₂ concentrations to about 600 p.p.m.v.) can thereby be lengthened by from 20 to 100 years².

Woods suggests that such a delay in temperature rise will ease the potential for traumatic climate change by providing extra time for initiation of measures to reduce climatic warming, such as the

accelerated development of nuclear in place of fossil energy. But this likely will not be the course of events. Rather, it is more probable that the needed remedial actions will be delayed until the observed climate change can be unequivocally identified with atmospheric CO₂ growth, by which time its concentration could have risen above a value whose ultimate equilibrium climate effect would be calamitous. From this perspective, thermal inertia of the oceans introduces a type of irreversibility which is obviously of major importance for policy, since global temperatures will continue rising even after a decision to drastically reduce fossil fuel use has been implemented.

Scientists' response to this dilemma is naturally to suggest that predictions, rather than observations of temperature change, be used as the basis for action. But this requires hard political decisions which are made more difficult by the acknowledged lack of understanding and quantifiability of the physical phenomena concerning climatic change and its impacts. How to properly bring the relevant but uncertain scientific information into the decision-making process, is important, and attention is starting to be paid to this type of need^{3,5}.

Before we conclude that existence of long thermal ocean inertia lags inevitably results in the major irreversibility dilemma presented above, we should point out a revision in the usual viewpoint that is needed in order to properly address the physics of the problem. The ocean transient response is normally described in terms of an e-folding time for approach to thermal equilibrium — setting atmospheric CO₂ level to be constant. However, from a policy viewpoint we need to follow the approach to a steady state following elimination of CO₂ emissions. All current carbon cycle models predict only a modest fall off rate of CO₂ levels following such a step⁶, but these probably do not allow for the involvement of deeper ocean layers that results in the extended e-folding times we have quoted. In fact, it seems intuitively clear than stronger coupling to the deep ocean implies a larger short-term oceanic sink for CO₂ deposition, and hence a faster fall of atmospheric CO₂ concentration following reduction of CO₂ emissions. If this qualitative surmise is true (and an analysis of it has yet to be made), the irreversibility feature of the policy issue we have raised may not be as severe as it seems at first glance.

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Misuse of statistics in social sciences

SIR—Two letters, one from Van Valen¹, the other from O'Quigley and Baudoin², have warned against the indiscriminate use of null hypothesis testing and the confusion of level of significance with scientific certainty. The situation is worse even than they make it seem, at least in the social sciences.

As Van Valen points out, R.A. Fisher developed the analysis of variance not for hypothesis testing but to aid scientific questioning. Instead it is used in the social sciences to come to scientifically irrelevant decisions about null hypotheses. My alpha (the level at which I reject the null hypothesis), cautious person that I am, is always one in a million. Yours is one in ten. We both live by the rules. You get a lot of significant results (or almost so, or highly so); I get none. Who is right scientifically?

Guttman³ argues that hypothesis testing as used in the social sciences is, strictly speaking, anti-scientific. Science is about the accumulation of information and testing by replication. Hypothesis testing is used to dodge replication and come to immediate decisions. Yet one test of significance leaves indeterminate the probability of the outcome of the next test on a new sample. In other words tests of significance are not cumulative and do not remove the need to replicate. The result is a landscape dotted with isolated studies forming no pattern and making no collective sense. Guttman also points out what is obvious on reflection, that simultaneous *F* tests on rows, columns, interactions and so on, routine in the social sciences and built into packages such as SPSS, encounter the same problem that simultaneous tests do; they are not independent. The same error flutter that makes one contrast improbable can affect another. The logic of hypothesis testing for any other than the one-way analysis of variance is flawed.

The Neyman–Pearson statistical tools are framed in terms of a loss function: the money or resources that a decision will cost. They are designed to minimize losses in gambling, farming, industry and war. It was gamblers who first demanded probability theory and agriculturists who adopted inferential statistics. Their common problem was the need to make decisions under risk. Social scientists, faced with difficult experimental problems, took up hypothesis testing *faute de mieux* to make progress rapidly and to lend their endeavours the appearance if not the reality of scientific respectability. It is in fact irrelevant to science which is concerned with making sense, not conserving resources.

The bankruptcy of programmes of research based on the crutch of the *F* test is revealed by the choice of the null hypothesis. It is almost always that a correlation or a mean difference is zero. If knowledge were accumulating the incumbent hypothesis would hardly ever be that of no correlation or no effect. Yet this is what is tested routinely, as if all that had gone before counted for nought. It is correct to be dubious about the claims of little old ladies to tell by taste how cups of tea were poured, milk first or second. We can calculate probabilities and find them out, just as we can detect fudging, by Mendel and by Burt. The hypothesis of no effect is the correct one for improbable claims, of psychokinesis or water divining, for example. But it is not correct when we have a body of knowledge to draw on and a theory designed to predict not just that something will happen but what and how much.

The fault does not lie with statisticians. Their job is to tell us how to do statistics, not science. It is scientists who have to work out how to do science. By misunderstanding the scientific use of statistical tools social scientists have created a situation every bit as bad as Guttman says it is. Puttering along, making the same old mistakes, will in the end bring the whole enterprise into disrepute.

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1. Van Valen, L. *Nature* 314, 230 (1985).
2. O'Quigley, J. O. & Baudoin, C. E. *Nature* 316, 582 (1985).
3. Guttman, I. *Statistician*, 26, 81–107 (1977); *Appl. Statistical Models Data Analysis 1* (in the press).

No to new photosynthetically active radiation units

SIR—In response to R.A. Lewin (*Nature* 316, 582; 1985), who proposes a new unit to measure photosynthetically active radiation, certain misconceptions should be dispelled. The photosynthetically active photon flux density (PPFD) is the quantity of photons incident on a unit area per unit time. It is not, as claimed, light intensity which is the luminous flux emitted by a point source into a unit solid angle (SI unit, cd), nor is it uniquely PAR (photosynthetically active radiation) which is commonly used for the radiant power (400–700nm) incident on a unit area per unit time (SI unit, W m^{-2}).

Communication between ecophysiologists has been greatly aided by the adoption of SI units. A particular advance has been the use of PPFD (units: $\mu\text{mol m}^{-2} \text{s}^{-1}$). This unit is consistent with those of O_2 , CO_2 and H_2O fluxes, greatly simplifying the calculation of derived parameters such as quantum efficiency of efficiency of water use.

There are limitations to the use of PPFD. Density is confusing for a flux through a unit area rather than volume. The term is biased to land plants being

limited to the photosynthetically active wavelengths (400–700nm). However, the 'alb', the new unit proposed by Lewin, solves none of these problems and only adds to the confusion.

Lewin also complains that PPFD requires the use of the Greek letter, μ , which is awkward to type. Awkward as it may be, modern scientific word-processing packages can easily solve this problem. In any case, SI derived units are composed of base units, thus the 'alb' would be $\text{mol m}^{-2} \text{s}^{-1}$, and avoidance of negative exponents, abhorred by Lewin, would require the use of the 'ualb'. Does the author object similarly to μg and μm ? Plants, especially algae, differ in their absorption spectra, but unless sensors can be designed to fit each individual plant, there is no practical solution to this problem, and the alb provides no advance. If Einstein's first name be abbreviated to alb, let it be for an advance in science and not for a unit which solves no problems and adds to the confusion of light measurements.

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SIR—To contradict Lewin's recent letter suggesting the alb as a radiation unit, instead of 'einstein', I consider that there is no confusion in the physics community about the use of the einstein. The ubiquitous ratio (velocity)/(velocity of light) = v/c is commonly known as β , and is otherwise not named. The authority for this statement is (1) your nearest friendly physics text, (2) excellent physicists, (3) one not-so-excellent physicist (L.X.F.).

Einstein's contributions to physics are incomparably wide and basic. So many units would be justifiably named after him than none is.

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Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a rather technical character which are not provoked by articles or letters previously published (where the Matters Arising section remains appropriate).

Healing in perversion

Michael Shepherd

The Breaking of Bodies and Minds: Torture, Psychiatric Abuse, and the Health Professions. Edited by Eric Stover and Elena O. Nightingale.

W.H. Freeman: 1985. Pp.319. Hbk \$21.95, £22; pbk \$11.95, £11.95.

TORTURE: "the art or process of inflicting severe pain, especially as a punishment, so as to extort confession, or in revenge". This is an impersonal dictionary definition of an unpleasantly personal topic, one which Voltaire saw as important enough to include in his *Philosophical Dictionary* of 1769 to underline its significance for the society of the day:

The Romans inflicted torture only on the slaves, but the slaves were not reckoned to be human. Nor does it appear that a judge . . . regards as a fellow man the haggard, pale, broken individual who is brought before him dull-eyed, with a long dirty beard, covered with the vermin that have been preying on him in the dungeon. He gives himself the pleasure of putting him to major and minor torture, in the presence of a surgeon who feels his pulse until he is in danger of death, after which they set to again. . . .

Until recently Voltaire's condemnatory views have been expressed by a relatively small minority, even though the twentieth century can claim some of the most savage examples of human barbarism in recorded history. On Human Rights Day in 1972, Amnesty International began its campaign against the systematic use of torture by governments, and in the following year published its disturbingly well-documented *Report on Torture* (Duckworth, 1973).

The *Report* included a full account of the medical and psychological aspects of torture and demonstrated by means of a survey that "torture has virtually become a world-wide phenomenon and that the torturing of citizens regardless of sex, age or the state of health in an effort to retain political power is a practice tolerated by others in an increasingly large number of countries". The topic has since attracted mounting attention and concern, though it was not until December 1984 that the Convention against Torture and Other Cruel, Inhuman or Degrading Treatment or Punishment was adopted by the United Nations General Assembly.

The psychological and psychiatric evidence, concluded the *Amnesty Report*, points to the existence of the potential to torture in *Homo sapiens*. Voltaire, who recognized torture as one aspect of the destructive element in man, would not have been surprised by these findings, while clinical observations have indicated that John Cowper Powys's admission that he could not remember a time "when sadistic thoughts and images did not disturb and intoxicate me" is unusual more by virtue of its frankness than by its con-

tent. Further, torture carries its own appeal. Writing of perhaps the most distressing scene in world literature, the blinding of Gloucester in Shakespeare's *King Lear*, Wilson Knight remarked shrewdly that, "The sight of physical torment to the uneducated brings laughter". And with the laughter goes a form of pleasure, widely exploited by pornographers and wholly unrelated to educational status.

The Breaking of Minds and Bodies, published under the auspices of the American Association for the Advancement of Science, should help carry the campaign against torture a stage further. Its subtitle, however, *Torture, Psychiatric Abuse, and the Health Professions*, is more informative than its title. The book is in two parts, one concentrating on general issues and the other on psychiatric practices in the Soviet Union, each containing five articles by various authors with several case-histories, along with editorial commentaries and three appendices providing codes of ethics, an inventory of relevant organizations and a selected bibliography. Much of this information is familiar and, indeed, three of the articles have appeared elsewhere. The principal purpose of the book, however, is not so much to record the activities of repressive governments as to explore how they have "enlisted the aid of medical practitioners in suppressing dissent and what steps need to be taken to prevent such professional complicity, and ultimately, to end the abuses themselves".

The message is all the more timely inasmuch as the involvement of the medical profession has not always been acknowledged. The medical profession, nominally devoted to the preservation of health and the saving of life, places great store on the ethical conduct of its members, as epitomized in the International Code of Medical Ethics, adopted by the World Assembly of the World Medical Association in 1949, which states categorically that "Under no circumstances is a doctor permitted to do anything that would weaken the physical or mental resistance of a human being except from strictly therapeutic or prophylactic indications, imposed in the name of his patient". In fact, as the evidence makes clear, medical practitioners continue to disobey this injunction, actively or passively, in many countries. Voltaire's pulse-taking physician, far from giving succour to the victims of torture, was participating in the process.

Why does this occur? The book focuses on psychiatrists in the Soviet Union, whose well-documented activities occupy a major part of the text. By employing their mental hospital system to coerce dissident but mentally healthy citizens and perverting clinical concepts for the purpose, the Russians have provided a new variation on an old theme. But anyone who has had contact with medical representatives of this system cannot fail to recognize, along with the cynical, unscrupulous apparatchik, the sincere, if misguided, believer in an ethical system codified in the Physician's Oath of the Soviet Union which, after mentioning the health of man and the care of disease, goes on to state: "I will in all of my actions be guided by the principles of communist morality, ever to bear in mind the high calling of the Soviet physician, and of my responsibility to the people and the Soviet state".

This chilling sentence draws attention sharply to the need for account to be taken of more than one set of ethical guidelines when assessing professional behaviour. As Heijder has pointed out (see *Professional Codes of Ethics Against Torture*, published by Amnesty International in 1976), such conduct is affected by at least four factors: political systems, public opinion, organizational units and individual codes of morality. The noxious doctor, like the immoral priest or the crooked lawyer or the dishonest scientist, is the product of all these forces, whose balance determines the answer to the question posed by a distinguished German-Jewish refugee who had left Germany early in 1933 after finding most of his colleagues at the university clinic dressed in Nazi uniform the day after Hitler's accession to power — in the event of such circumstances in this country, he would ask, how many of your colleagues would be wearing civilian clothes?

Clearly it is unnecessary to postulate a Joseph Mengele in the vast majority of those uniformed physicians. The central issue is how far association with or acceptance of any system which employs or condones torture in any form is compatible with a medical ethos which must be preserved if the profession is to maintain its public status and its self-respect. Stanley Milgram's disturbing experiments on obedience and disobedience in response to authority reinforce the fragility of personal conscience in conflict with social pressures and reinforce the verdict of the *Amnesty Report*, namely that "The prevention of torture . . . lies not in medical research but in political and legal remedies". The value of this book resides in its contribution to the heightening of public awareness and the facilitation of official action. □

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Between a good read and a good reed

John C. Marshall

Children's Reading Problems. By Peter Bryant and Lynette Bradley. Basil Blackwell: 1985. Pp.116. Hbk £16.50, \$24.95; pbk £5.95, \$9.95.

Reading Ability. By Charles A. Perfetti. Oxford University Press: 1985. Pp.282. £25, \$35.

In 1945 Herbert Hoover declared that the liquidation of illiteracy was one of the five or six major tasks for the postwar world. It still is. Even in the industrialized world a small but distressing minority of children experience great difficulty in learning to read and never achieve the fluent command of visual language that is necessary to participate fully in a technological society; what can be done for the 'slow learner' or the 'dyslexic' child? Many students in tertiary education who have mastered the nuts and bolts of reading complain of slow reading speeds and poor comprehension; can these young adults learn to cope better with the demands that professional life places upon true literacy?

These two books reach a similar conclusion on one key issue: the invention of the alphabet was a good idea, and a firm grasp of the alphabetic principle that letters (or letter groups) map onto the constituent sounds of words is of crucial importance in learning to read. Beyond this, the books differ greatly in both style and content.

The primary virtue of Bryant and Bradley's monograph lies not so much in their conclusion *per se* (advocates of so-called 'phonic' instruction have been with us for a century or more), but rather in their evidence for that conclusion. First reported in this journal (*Nature*, 310, 419–421, 1983), Bradley and Bryant have provided the most compelling demonstration that awareness of the relationship between letters and sounds is *causally* implicated in the efficiency with which children learn to read.

Children's Reading Problems contains a vigorous, albeit good-natured, attack upon the methodological and interpretative inadequacies of much previous research; it defends, more cogently than any other work I know, the central importance of phonic skills. The book is written in lucid English, quite free of psychological jargon. Parents and teachers should find Bryant and Bradley's simple description of their training methods of considerable practical value. More generally, Bryant and Bradley provide an acute analysis of how to conduct serious educational research on which practical decision-making could be rationally based.

One aspect of their work is, however, controversial. They fail to acknowledge the full import of the fact that English (or

French) cannot be read or written solely on the basis of letter-sound correspondence rules. The child who writes, YOT, or PLAZE (for "yacht" and "plays") has mastered the skills that Bryant and Bradley stress; likewise, the child who reads SHOE as "show" or SWEAT as "sweet". But to attain mature literacy the child must eventually come to read and write orthographically and forgo reliance on phonic rules; a good read is not the same as a good reed. One alarming consequence of Bryant and Bradley's over-reliance on phonological awareness is their belief that reading backwardness must have a solitary cause — failure to acquire normally the regular mappings between letters and sounds.

This flies in the face of the extensive evidence, from single-case studies, that backward readers (or 'developmental dyslexics') constitute a very heterogeneous population with striking qualitative variation from child to child in the nature of their impaired and intact sub-skills. Bryant and Bradley are well aware of this literature (they quote a few pertinent examples), yet they dismiss its significance on the peculiar grounds that analogous qualitative variation can be found in younger children who read at age level. This seems such an obvious *non-sequitur* — why should the balance of skills required to read normally at one age be identical with that required for normal reading at a later age? — that one is deeply puzzled to see it put forward by *developmental* psychologists.

By contrast with the straight but narrow path chosen by Bryant and Bradley, Perfetti reviews a substantial chunk of the primary literature on all aspects of reading ability in both children and adults; the discussion ranges from word recognition to text comprehension, from the analysis of eye movements in skilled reading to the role of verbal short term memory, and from brain laterality to computerized reading instruction. Many of the studies

reported are as relevant to speech processing and oral comprehension as they are to reading and writing. These review chapters are solid and workmanlike, although somewhat heavy going; Perfetti's turgid style lacks the sparkle and excitement of Bryant and Bradley's clear arguments.

Perfetti's own "verbal efficiency theory" succeeds in showing that comprehension of a written text is a complicated business but provides little real insight into the mysterious processes of "propositional assembly" and "propositional integration". Much of this research has a curiously circular flavour. Groups of high- and low-ability readers are selected and then shown to differ on a variety of reading-related skills. The unproductiveness of such a strategy derives in part from the use of heterogeneous groups; it is thus unclear whether a particular impairment is characteristic of all low-ability readers or solely that of a subgroup. Many of the studies also fail to distinguish between cause and effect; an impairment of "verbal intelligence" could be a cause of poor comprehension, but equally the failure to learn from reading may be reflected in poor performance on other language tasks. In contrast, Bryant and Bradley's discussion of how to disentangle cause and effect is particularly incisive.

These problems assume major significance in Perfetti's chapter on dyslexia. He tentatively concludes that "dyslexia may be the low end of an ability continuum rather than a qualitatively different problem." But once again, the failure to distinguish individuals from groups renders the conclusion suspect. Indeed, the very concept of a single ability continuum seems misguided when we are dealing with cognitive functions, such as reading and writing, whose effective deployment depends upon a large range of subcomponents. □

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IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

Some mistake? Thomas Bennett's "Eureka" driving wheel for bicycles of 1897, which, he boasted, made "practically a frictionless machine". The illustration is reproduced from *A Victorian World of Science*, by Alan Sutton, published by Adam Hilger price £12.50, \$20.

Into the generation of application

Karol Sikora

Hybridoma Technology in the Biosciences and Medicine. Edited by Timothy A. Springer. Plenum: 1985. Pp.602. \$75, £71. **Human Hybridomas and Monoclonal Antibodies.** Edited by E.G. Engelman, S.K. Fount, J. Larrick and A. Raubitschek. Plenum: 1985. Pp.526. \$69.50, £66.

SINCE the discovery ten years ago of monoclonal antibodies, several books about these biologically useful tools have been produced. Until now, however, they have tended to concentrate heavily on methodology. But we are beginning to see the emergence of second generation volumes which provide the reader with much more sophisticated detail on the application of the technology. These two books are in this category.

The first, edited by Timothy Springer from Harvard University, starts with a rather dry title. However, in it the editor has managed to get together a group of authors who take us through some of the more elegant approaches to the construction and characterization of monoclonal antibodies. Recent developments in hybridoma technology involved in the production of monoclonal antibodies — peptide immunization, the expression of cloned immunoglobulin genes, and exon shuffling and class switching — are all considered and fully referenced. I know of no other source to date where this information can be found in one place. The merger of gene cloning and the monoclonal antibody fields promises to bring mutual benefit. Designer monoclonal antibodies — that is those produced with the required secondary functions by recombinant gene technology, may now become a reality, whilst the speed of cloning defined genes can be aided by having an appropriate antibody to the resulting protein.

The second section consists of a series of reviews on the biomedical application of monoclonal antibodies. Some of these are rather unoriginal and now dated, especially those concerning clinical applications. The final section considers T-lymphocyte clones and their products. Unfortunately this field has moved too fast for the publisher and although there are some useful background reviews little recent information is to be found. Altogether this is an interesting volume, containing much useful reference material about new ways to produce and improve monoclonal antibodies, but it could easily have been shortened without detriment by removing some of the reviews on specific applications.

The production of human monoclonal antibodies has captured the imagination of many over the past five years. Their

advantages in allowing a direct examination of the human immune system and the potential lack of immune response to them on injection suggest that they will be more useful clinically than their rodent counterparts. But there are tremendous technical difficulties in their production and these problems are addressed by the contributors to the first volume specifically to be concerned with human monoclonal antibodies.

The technical problems and how they can be overcome are considered in the first part of the book, which provides recipes for mouse-human and human-human hybridization as well as the use of Epstein-Barr virus to achieve immortalization. The production of human antibodies to cancer cells, infectious agents and self antigens in autoimmune disorders such as diabetes and systemic lupus erythematosus are reviewed. An interesting mixture of chapters then considers specific aspects such as the study of human-human hybridomas in immunodeficiency, the use of electrofusion techniques and the generation of human

T-lymphocyte hybridomas. There follows an appendix listing methods for tissue culture and assaying supernatants and recipes for media. Lists such as these have little to do with human monoclonal antibody production specifically and are readily available elsewhere; so, again, the book is unnecessarily long despite having a useful summary of current work in this difficult area. The considerable interest of the biotechnology industry here, together with recent developments in handling and expressing human genes, will almost certainly ensure rapid development of this field.

Now that monoclonal antibodies are impinging on wide areas of biological and clinical science there is a great need for books to function as knowledge conversion kits similar to those available for upgrading construction toys for children. This would save readers considerable time and money. □

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Journey to adulthood

Leonard A. Freed

The Growth and Development of Birds. By Raymond J. O'Connor. Wiley: 1984. Pp.315. £22, \$34.15.

BIRDS are extremely varied with respect to the size and composition of their eggs, the nature of the nests they provide for eggs and neonates, and growth and development both during incubation and after hatching. They range from species without parental care following egg-laying to those in which care continues to long past the fledging stage.

Growth and development occur in: (i) a thermal environment influenced by nest, parental brooding, brood size, and maturation of individual endothermy; (ii) a nutritional environment influenced by diet and frequency of feedings; (iii) a social environment in which there is parent-offspring interaction, sibling rivalry for parental attention, and interaction with conspecifics during dispersal, migration and recruitment for breeding; and (iv) a demographic environment with risks of mortality at each life-history stage. The nature and combinations of these environments differ between taxonomic groups (indicating phylogenetic constraints on developmental patterns), as well as within them for species in different ecological circumstances (indicating evolutionary adaptation of developmental patterns).

Raymond O'Connor's *The Growth and Development of Birds* is an important first review of aspects of these environments through its attempt to combine physiological, behavioural and ecological studies

within an evolutionary perspective. The book is structured around the avian life-cycle, bringing together discussion of an unusually wide range of topics — including those mentioned above — and comparing altricial and precocial species for many of them. The goal of the book is clearly breadth rather than depth of coverage. But while specialists may be disappointed by the absence of certain details to do with their particular research interest, or the scarcity of work published after 1980, they will undoubtedly be stimulated by seeing their area put in context and perhaps moved to examine aspects of it they might previously have overlooked.

Readers should be aware that several topics (for example asynchronous hatching and optimization of growth rates) have become increasingly controversial since the book was written, and also that most examples are based on North Temperate species. Tropical birds, with different life histories and social structures, remain understudied in all aspects of growth and development. Nevertheless, the book will be read with profit by a wide range of biologists, and beginning graduate students and undergraduates will here encounter features of growth and development that they would be unlikely to come across in any single course. □

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● Academic Press has just published the third volume in the series *Form and Function in Birds* edited by A.S. King and J. McLelland; the emphasis in this volume is upon locomotion and the special sense organs. Price is \$99.50, £90.

Out of space, out of time

John D. Barrow

Relativistic Astrophysics. By M. Demiański. Translated by A. Pol. Pergamon: 1985. Pp.341. £25, \$45.

RELATIVISTIC astrophysics means different things to different people. To some it is special relativistic astrophysics with an emphasis upon the physical complexities of high-energy radiation processes, pulsars, accretion disks and neutron stars; to others it means general relativistic astrophysics and the exotic realm of ultra-strong gravitational fields; black holes, gravitational collapse and gravitational waves. Marek Demiański's text is of the general relativistic sort.

The author assumes the student possesses a basic knowledge of general relativity and tensor calculus, the basics of which are completed by page four. The book then commences proper with a succinctly written study of black holes, including a real derivation of the Kerr solution via the Ernst potential and a study of its orbits; derivations of the laws of black hole mechanics and thermodynamics together with simple analyses of the stability of the Kerr and Schwarzschild black hole solutions come later.

Between these discussions of areas which have been of special interest to the author is sandwiched a less compelling section of relativistic hydro- and thermodynamics, with a heavy emphasis on for-

malism that in subsequently put to little use. But good chapters follow on the stability of isolated self-gravitating bodies in Newtonian and general relativistic gravity with a detailed discussion of the effects of rotation. There are less quantitative surveys of astrophysical processes in the vicinity of black holes and neutron stars but these fall short of the standards set by other recently published textbooks — in particular that of Shapiro and Teukolsky's *Black Holes, White Dwarfs and Neutron Stars* (Wiley: 1983).

The final chapter, devoted to cosmology, is very disappointing. Here, the fact that the text was first written in 1978 finally catches up with the publishers. Although the earlier chapters on mathematical aspects of relativistic astrophysics have not dated during the prolonged translation period, the subject of cosmology has been revolutionized to such an extent that the author's discussion of physical processes in the early universe and galaxy formation is seriously out of date. The most recent references in the bibliographies are to works published in 1978 and 1979.

As the starting point for a second course on general relativity and its non-cosmological applications this book can be recommended to students for its systematic and lucid exposition, but the reader who is interested in contemporary ideas in astrophysics and cosmology will find that the story told by Demiański has been rather overtaken by Old Father Time. □

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wide range of sectors. The analysis therefore in either scholarly or policy terms is rather unsatisfactory.

They provide a good introduction to the macro-economic literature on informatics and industrialization, employment and the comparative advantages, for example. But it ought to have been set out in a clearer more coherent manner. It is, for example, impossible to make quick reference to tables, which are not well labelled. The authors use a curious character coding which is difficult to follow because the key is to be found on Table 6.2, which is mentioned without giving a page reference. Books ought by first principles to be easily read and understood. And for students, hungry for material on the subject, the splendidly researched literature review is a hesitant and much qualified academic discussion rather than a clear indication of controversy or major contributions to knowledge.

Stacking the Chips works on a model developed by Cole in 1982 which demonstrated that technological change produced significant employment and distributional effects. This book, by its own description, attempts a very crude interpolation of available research evidence, despite Raphael Kaplinsky's warning in *Microelectronics and Employment Revisited* (ILO, Geneva, 1985) about the major gaps in coverage, methodology and analysis. After reading this latest contribution, I conclude that much indeed still needs to be done.

The conclusion that new technology does not necessarily lead to job destruction in the long term, but that shorter term structural problems are evident, repeats old caveats, many of which were pioneered at the Sussex University Science Policy Research Unit. With the exceptionally broad brief chosen by the authors, the obvious can only be reaffirmed. If in future they are at all interested in studies dealing with fewer technologies, sectors and a more modest terrain than the global economy in eight segments, I look forward to the sequel. □

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Going through the changes

Rita Cruise O'Brien

Stacking the Chips: Information Technology and the Distribution of Income. By J. Bessant and S. Cole. Frances Pinter, London/Rowman & Allanheld, 81 Adams Drive, Totowa, New Jersey: 1985. Pp.290. £17.50, \$26.95.

GETTING beyond the generalities and hyperbole in discussion of the effect of information technology on developing countries has been an uphill struggle. At a recent meeting of the North-South Roundtable (Society for International Development) on this topic, which included leading government spokesmen, scholars and business people, it became clear that some progress had been made. For some years the approach "Information Technology: Blessing or Plague of the Third World" prevailed making it difficult at times to make a sensible contribution.

Central to researching or writing about

informatics and development are questions of opportunity cost, technology transfer, income distribution and employment effects. I prefer to consider small corners within the field such as the experience of a single country or a particular problem which might be illustrative of others. For example, was it a wise government directive to have automated the Brazilian banking system, making banks more efficient at international dealing and trading, while destroying many jobs at the clerical level inside the country's banks? The authors of *Stacking the Chips* however, have opted for comprehensive analysis and refinement of economic modelling on the subject.

The authors did their work at the Sussex University Science Policy Research Unit (a centre of excellent research both on industrialized and developing countries) on a grant from the UN Institute for Training and Research. Ostensibly the book is about the effects of high technology on the distribution of income. The authors obviously felt it necessary to clear a lot of the ground first but instead of then delving more deeply into the subject, they introduce many different variables covering a

New editions

- *Ancient Sedimentary Environments (and their sub-surface diagnosis)* 3rd Edn. by R.C. Selley. Publisher is Chapman & Hall. price is hbk £27.50; pbk £10.95.
- *Color Measurement: Theme and Variations* 2nd Edn. by D.L. MacAdam. Publisher is Springer-Verlag. price is pbk DM 94.
- *Biotechnology: A New Industrial Revolution* revised edition, by Steve Prentis. Publisher is Orbis, London. price is £12.
- *Seismic Migration: Part A, Theoretical Aspects* 3rd Edn. by A.J. Berkhout. Publisher is Elsevier. price is Dfl.190, \$70.50.
- *Cryogenic Systems* 2nd Edn. by Randall F. Barron. Publisher is Oxford University Press. price is £60. This book may be available in the United States, but the publisher has been unable to provide that information.

Small-scale processes in the upper ocean boundary layer

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Here I review some of the recent advances in our knowledge of the physics of the upper ocean boundary layer at scales of micrometres to tens of metres, with emphasis on the nature of turbulence in the layer. A study of sub-surface bubbles produced by breaking wind waves offers some hope of revealing more of the turbulent processes, and indicates that bubbles may be important in the transfer of gases from air to sea.

OCEANOGRAPHERS are presently paying increased attention to the small-scale processes which operate at, or near, the sea surface. With rare exceptions, interest has been dormant since the exciting era in the 1950s when people such as Woodcock¹ were actively involved in seeking solutions to questions of air-sea interaction which required information about the small-scale physics, notably those relating to the production of spray and aerosols. As often happens, it has partly been the development of novel instruments which has revived this area of interest. The use of microwave satellite sensors—the synthetic aperture radar and scatterometer—and surface X-band radar has prompted new questions about the small-scale topography of the sea surface to which the instruments are sensitive. These questions must be answered if we are to make effective use of the measured data, perhaps to infer wind stress from the scatterometer by relations which are better than empirical, and hence improve the representation of atmospheric forcing in global models of ocean circulation. Better estimates of air-sea gas fluxes are required for global budget models and, for example, to enable oceanographers to use tracers such as tritium (with helium decay) to date water masses which lose contact with the surface and pass into—and ‘ventilate’—the main thermocline.

Some of the answers are already becoming available (in particular, studies² have been made of microwave scattering from breaking waves). Even the perennial questions of the role and importance of breaking waves in the transport of momentum from air to sea and of their generation of turbulence in the sea itself are close to being answered in a quantified way^{3,4}, although we still require a clear explanation of some puzzling but well-known phenomena, for example why it is that whitecapping is less frequent when it is raining than when it is not, other factors (for example, wind speed) remaining the same.

The fundamental question which remains to be answered is ‘What is the nature of turbulence below the sea surface?’ This

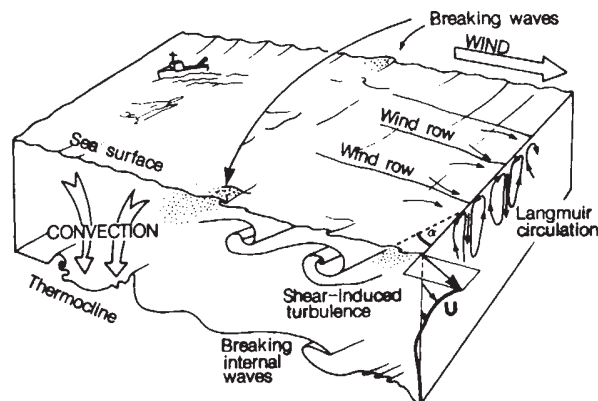
question concerns the ‘mixed layer’ or, to avoid ambiguity, the ‘upper ocean boundary layer’ (either term may include the seasonal thermocline). Some of the mechanisms affecting the turbulence are illustrated in Fig. 1. Moisture flux and radiative effects, essential in studies of convection, will be mentioned only in passing. I have also not discussed fronts; they are usually larger than the scales that I have chosen to describe.

Ocean/atmosphere similarities

It has frequently been assumed that the dynamics of the upper ocean boundary layer are similar to those of the atmospheric boundary layer. Indeed, studies of both mean quantities and fluctuations provide evidence in support of this hypothesis, although without exception the volume of data which is available for comparison is sparse and scarcely sufficient to confidently establish a similarity.

The mean flow in the upper few metres of the ocean and lakes has been measured by Churchill and Csanady⁵ in neutral conditions (those in which atmospheric heating or cooling is negligible). The near-logarithmic variation of speed with depth found by observing the motion of floats is similar to that of the wind in the atmosphere. Two of the authors’ conclusions are particularly remarkable. The ‘roughness length’ in the water (a length scale found empirically from the observations and, as the name implies, some measure of the nature of the surface) is some two orders of magnitude greater than that in the air (of the order of tens of centimetres rather than a few millimetres). Second, in conditions when the wave field was strongly affected by swell, much higher values of friction velocity, u_* (equal to the square root of the stress divided by the fluid density), in the water were inferred than could be explained by a conventional wind-drag relationship. The authors suggested, however, that this might be accounted for by the greater importance in these conditions of the wave-induced mean drift in the water—the

Fig. 1 Some of the mechanisms affecting turbulence in the upper ocean boundary layer. The spiral-like structure of the mean current (U) is due to the Earth’s rotation and reverses in sense in the Southern Hemisphere. The orientation of the ‘billows’ labelled ‘shear-induced turbulence’ is determined by the mean shear, so that their (horizontal) axes are not at right angles to the wind (that is, on average, angle $\alpha > 0$ in the Northern Hemisphere). Breaking surface waves and Langmuir circulation are, in particular, processes responsible for redistributing floating particles or biota. Breaking internal waves are included in the diagram, although they are not discussed in the text; they may be important in affecting the flux of nutrients and occasionally have an effect at the sea surface itself (see ref. 41). The wakes of ships will rarely be significant contributors to the net fluxes, except with respect to low-frequency sound!



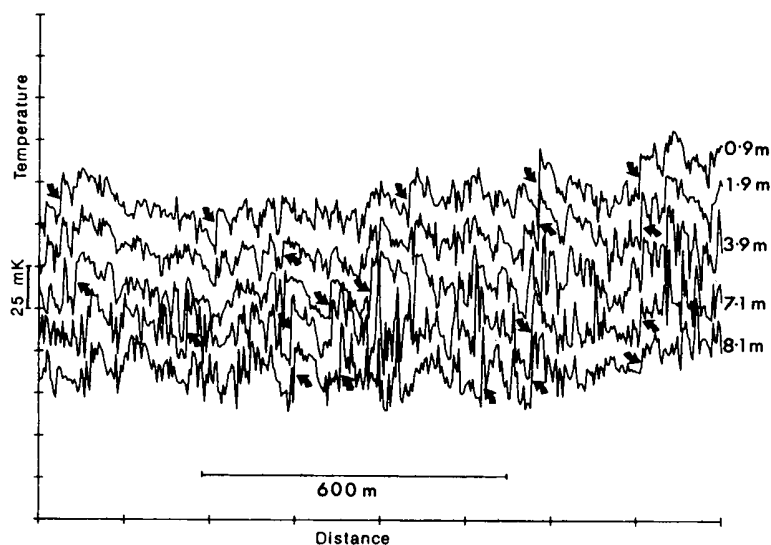


Fig. 2 Temperature fluctuations measured at the indicated depths from a spar hanging beneath a towed catamaran near 38°40'N, 12°50'W in October 1984 in daytime under clear skies. The wind speed was $\sim 7 \text{ m s}^{-1}$. Time variations have been converted to distance using the mean towing speed through the water, 1.9 m s^{-1} . Some of the abrupt temperature rises are marked by arrows.

'Stokes drift'. (The use of floats, or 'drifters', to measure drift currents may, however, be less than ideal because of the higher than average horizontal currents found below wind rows towards which floating objects tend to be drawn; I shall address Langmuir circulation below.) Flows in non-neutral conditions have yet to be closely studied in the ocean. However, it is known that the diurnal heating cycle can have a profound effect in modulating the oceanic near-surface stratification and currents, just as it does in the atmosphere⁶. A diurnal modulation of the current in the near-surface ocean, a variation resembling the well-known nocturnal jet in the atmosphere⁷ but being essentially a daytime event, has been observed⁸ and modelled^{8,9}; it results from the daily cycling of the layer depth into which wind-driven momentum is distributed as the stratification varies due to solar heating, and the subsequent inertial development of the current under the effect of the Earth's rotation.

Turbulence in the atmosphere is driven by convection and by the stress of the wind on the underlying boundary. In the ocean, turbulence may be similarly driven by convection and by wind stress, although less directly as waves act as an intermediary (see below). In the case of turbulent motions in near-neutral conditions, a similarity of the horizontal velocity spectra in lakes and ocean has been found¹⁰ when they are scaled with depth, z , and friction velocity. The spectra collapse into the canonical spectral form of flow in a turbulent boundary layer, as determined by laboratory experiments (ref. 11; see also ref. 12). The scaling of the rate of dissipation of turbulent kinetic energy in the ocean, ϵ , with the cube of the wind speed¹³ and inversely¹⁴ with depth provides more support for constant stress layer similarity, but these are relationships which deserve further investigation. Such investigation is now possible through the development (notably by Dillon; private communication) of upward-profiling turbulence probes capable of measurements right up to the sea surface. The most convincing demonstration of similarity between the oceanic and atmospheric boundary layers is perhaps the study of convective conditions in the ocean¹⁵ and the comparison with atmospheric data¹⁶. It is found that in both the ocean and the atmosphere, ϵ is uniform throughout much of the layer where convection dominates over the effects of surface stress (that is, beyond a Monin-Obukov length from the surface) and is proportional to the effective flux of buoyancy due to the heat passing through the surface. It is only near the surface that differences between the atmosphere and ocean appear significant (that is, $z/D < 0.3$ in the comparison of ref. 15, where D is the depth to which convection extends, here $\sim 150 \text{ m}$). The turbulence probes used in that study are now being used to address fundamental questions about the turbulent structure of stratified fluids and to obtain the measurements of turbulence and diffusion in the upper ocean that are

required for numerical models of the ocean (see, for example, ref. 17).

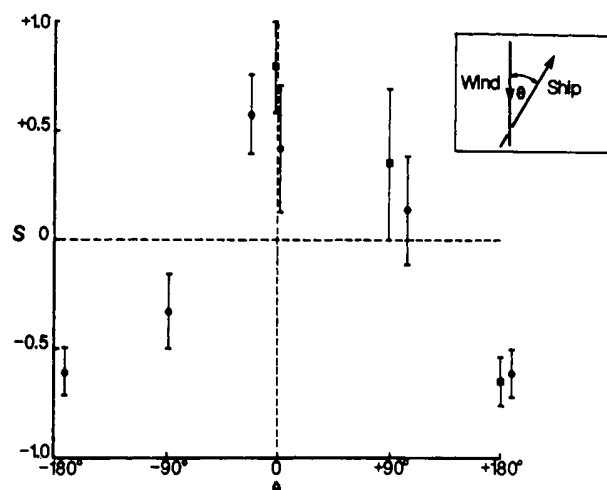
One other measurement in which similarity is found between ocean and atmosphere is that of temperature structure. Temperatures measured at fixed positions in the atmospheric boundary layer are found to have a 'sawtooth' pattern, with rather abrupt changes (predominantly in the same sense in given conditions of heat flux) being followed by relatively gradual ramps, thus leading to skewed temperature derivatives¹⁸. Similar skewed gradients have been reported from towed or moored instruments in a lake¹⁹, and have now been observed at sea (Figs 2, 3). In conditions in which the heat flux leads to stable stratification, the skewness measured in upwind tows (or from moorings) is positive; it reverses sign in downwind tows. Intriguingly, the skewness usually appears to be slightly positive in tows made to the right of the wind ($\theta = 90^\circ$ in Fig. 3; this is reversed in tows to the left of the wind), presumably because the Ekman flow, the rotation of the current vector with depth due to Coriolis forces, leads to a pattern of eddies or 'billows' which are, on average, rotated as sketched in Fig. 1 (the features labelled 'shear-induced turbulence'). If so, the trend should reverse in the Southern Hemisphere. It has not yet been established that inverse temperature ramps and skewness of the opposite sign are found in convectively unstable conditions in the ocean, but this may be expected from experiments in the laboratory and in the atmospheric boundary layer²⁰. Specifically, the sign of the skewness of the streamwise temperature gradient, sign S , is given by sign $(\mathbf{x} \cdot \nabla T \times \boldsymbol{\omega})$, where $\mathbf{x}$ is a vector in the flow direction, ∇T is the gradient of the mean temperature and $\boldsymbol{\omega}$ is the vorticity of the mean flow.

The existence of temperature ramps—and these have some coherence in the vertical (see Fig. 2)—suggests the presence of organized structures²¹; this is not surprising as large coherent structures are now known to be a feature of turbulent shear flows. We need, however, to discover whether significant vertical fluxes of heat and momentum are carried by these structures in the ocean for, if this is the case, it might be possible to devise a semi-deterministic model of the turbulence by specifically accounting for their presence. The construction of such a model is feasible, especially if, as is possible, the flow is close to marginal stability in conditions of stable heating^{22,23} and the ramps are related to the most 'dangerous', or—once instability occurs—most rapidly growing modes.

Langmuir circulation

This brings me to comment briefly, but emphatically, on the near-surface organized structure consisting of alternating vortices aligned downwind which are known as the Langmuir circulation. This is well known to produce a coherent structure

Fig. 3 Variation of the skewness of the temperature derivative, S , measured in tows at constant depth at different angles, θ , to the wind when the net air-water heat flux is positive. The circles represent measurements made at sea (for example, in the Northern Hemisphere, as in Fig. 2) and the squares represent measurements from Loch Ness¹⁹. Corresponding values of S at $\theta = 0$ in the atmospheric boundary layer are in the range 0.4–0.85 (refs 18, 20).



at the sea surface—the associated wind rows into which floating material is carried—and, as Leibovich²⁴ has suggested, we might also account for its effects on the fluxes below the surface in a deterministic way.

It is a profound embarrassment to physical oceanographers that we have not yet established whether, or when, Langmuir circulation is important in distributing heat and momentum in the upper ocean or in forming the seasonal thermocline. This has remained one of the most challenging but well-defined tasks in physical oceanography since Langmuir wrote his definitive paper 47 yrs ago⁴². Recent observations made from the ocean research platform Flip²⁵ have demonstrated that, in addition to the enhanced downwind velocities which I mentioned above, remarkably large downward velocities can be measured beneath wind rows. The vertical momentum carried by these circulations—the associated Reynolds stress—appears too great in some of the cases studied to be in equilibrium with the local wind, suggesting a considerable variability in structure; more observations are required to link measurement to theory. Whether the circulation has an immediate counterpart in the atmospheric boundary layer depends on how it is generated or on which theory one accepts. The most plausible theory (in that it best satisfies the existing observations) is that development by Craik and Leibovich²⁴. The pattern of streamwise vortices is generated by the interaction of waves and mean shear. Some of the theoretical predictions have been verified in the laboratory²⁶. We know the mechanism works, but is it the source of circulation in the ocean, and can theory be used to predict the associated fluxes?

Breaking waves

The differences between oceanic and atmospheric boundary layer dynamics are due partly to radiative and evaporative processes, but also to the asymmetrical nature of the sea surface, the turbulence generated by breaking waves, and the relative importance of mean flow, wave-induced motions and turbulent fluctuations. A fundamental asymmetry is found in the shape of surface waves; they have narrow crests and relatively broad troughs. The formidable nonlinearity of the boundary condition at the free surface makes the theoretical study of wave instability and of the onset of breaking one of great complexity, yet considerable advances have been made (see, for example, ref. 27). It has long been known that breaking occurs at or near the wave crests; this results in the production of sub-surface bubbles and of foam sometimes compacted into a layer many bubbles deep. Spray, the antithesis of bubbles, is thrown into the air, but whilst the opposite of foam—drops of water surrounded by air but resting on the interface—does occur briefly, the drops are very unstable and do not build up into compact layers. It is almost frivolous to mention that rarely is there in the ocean a deep source of rising bubbles; the antithesis of rain is rare. One

particular feature pertinent to the role of bubbles in contributing to the gas flux across the sea surface is that they have only to be carried down to a depth of ~ 10 m before their internal pressure is doubled hydrostatically. Spray, on the other hand, has to ascend to over 5 km before it experiences a reduction in pressure to a half of that at its origin at the sea surface. The wind speed at 10 m above the sea surface, W , is usually comparable to the dominant wave speed, c . The turbulent and wave-induced fluctuations in the air are comparable in magnitude, but are generally less than the wind speed. In the near-surface ocean, however, the wave speed and the wave particle velocities usually greatly exceed the mean flow and the turbulent fluctuations. Motion is dominated by waves. The critical layer where the wave speed matches the mean flow occurs in the air rather than in the water. (The water, however, generally carries the greater momentum per unit volume, and has a much greater heat capacity.)

The development of multiply connected surfaces as waves overturn and fluid re-enters through the water surface, allows the waves to generate vorticity (a characteristic of turbulent motion) even in a pre-existing irrotational flow; the conditions (described by Kelvin's circulation theorem) in which vorticity is conserved are violated. There are several possible candidates responsible for producing turbulence in breaking waves, perhaps the most obvious of which is instability at the head of, and around, the injection jet at the tip of a plunging wave after its entry into the water surface. A second candidate is the wake region of large rising bubbles, especially spherical-cap bubbles, entrained by a breaking wave. But a more potent and frequent source in both plunging and spilling breakers appears to be the shear layer formed between the spilling water of the breaker zone moving at about the speed of the wave crest and the underlying water moving backwards relative to the crest. The instability of this shear zone may be the source of oscillations in the spilling zone of breaking waves observed by Banner and Fooks².

Very little is known of the nature (for example, spectra and patchiness) of wave-induced turbulence, although this is becoming an active area of research. Some recent advances, especially those in laboratory experiments, are reviewed in the proceedings of a conference held recently in Japan²⁸. Most of the measurements of velocity fluctuations very near the sea surface have been concerned primarily with those at, or near, the wave frequencies. Early measurements at higher frequencies²⁹ concluded that almost all the turbulent energy produced by waves must be dissipated in the upper few metres, whilst Russian workers³⁰ found that the near-surface Reynolds stress decreased with depth and appeared to be associated with wave frequencies. Kitaigorodskii *et al.*³¹ have recently analysed measurements of turbulence made with a drag sphere mounted on a lake tower, and conclude that there is intense wave-generated turbulence

in the region immediately below the surface, of thickness about 10 times the mean wave amplitude, ζ , whilst at greater depths the turbulence has the character of the conventional constant stress layer. The region of wave-generated turbulence is quite thick: values of ζ of 2 m, which is not uncommon, imply that the region extends to 20 m below the surface! Further work is required to substantiate these observations.

Bubbles and turbulence

Clouds of sub-surface bubbles produced by breaking wind waves can be detected by narrow-beam upward-pointing sonars moored or mounted on the sea bed^{32,33}. The mean depth to which these clouds of bubbles extend, $\bar{d}$, non-dimensionalized with wavelength, λ , increases with the ratio of wind to wave speed, W/c ; typically, in open sea conditions, $\bar{d}$ reaches 10 m for winds of $\sim 14 \text{ m s}^{-1}$. The acoustic scattering cross-section of the bubble clouds at a fixed depth, M_v , a measure of the approximate area of the bubbles per unit volume when the sonar frequency is sufficiently high, increases rapidly with wind speed (roughly as W^{12}) but decreases roughly exponentially with depth with a decay scale of 0.5–2 m. In high winds the status layer of bubbles formed below the surface can be dense enough to muffle the ambient noise in the sea produced by breaking waves³⁴.

The challenge is to solve the inverse problem—to obtain information about the turbulence from the measured vertical distribution of the bubble clouds; this has been attempted by comparing the observed distribution with predictions from models based on a balance between turbulent diffusion, the upward buoyant rise of bubbles of a given radius, and the flux in radius space due to bubbles being compressed hydrostatically as they are carried downwards or by their exchanging their constituent gasses with those dissolved in the water. Several assumptions made in the models raise further interesting questions about the turbulent dynamics near the sea surface and the analogies between water droplets in the atmosphere and bubbles in the sea. Various representations of turbulence have been tried³⁵, the most successful being that of adopting an eddy diffusion coefficient, K_v , of the classical boundary layer form, ku_*z , where k is Von Karman's constant. The values of u_* required for agreement with the observations are, however, greater than those derived from the wind speed, assuming a typical drag coefficient and continuity of stress across the sea surface. The ratio is close to unity for W/c near 2, but increases to ~ 2.5 for $W/c = 0.8$, when swell is of greater importance in the wave field. This result, reminiscent of Churchill and Csanady's⁵ conclusions based on drift currents, deserves further critical and careful investigation. A fit using a form of K_v which tends to a constant value in the surface layer but tends to the conventional ku_*z at depth, and which assumes that u_* is given by the wind drag, gives a mean surface layer thickness of 9.7ζ ,

which is intriguingly close to the conclusion of Kitaigorodskii *et al.*³¹ concerning the region dominated by wave turbulence.

The temporal variation of bubble clouds at a fixed position (and this is what is measured by a moored sonar) can be linked via advection and the lifetime of bubble clouds to the spatial and temporal distribution of the breaking waves which create them, and so to the whitecap area. Here there is the prospect of linking bubbles, turbulence, waves and foam to the questions of momentum transfer³⁶. We are again moving towards more deterministic models of forcing the turbulence in the sea; momentum is injected in pulses when wave groups break at a rate just in balance with the wind-forced growth of waves between breaking events^{3,4}.

Gas transfer

I must finally mention gas transfer because this is another area of upper ocean research in which significant advances are being made. Kitaigorodskii³⁷ has directed attention to the importance of patches of enhanced turbulence generated by breaking waves in changing the thickness of the viscous sublayer at the surface across which gases are exchanged with the atmosphere. Others^{33,38,39} have pointed to the possible importance of bubbles. As bubbles are carried beneath the surface, their internal pressure increases considerably and this acts like a piston to drive gas into the surrounding water, at least until the water itself becomes supersaturated. The equilibrium condition is thus one in which the water is supersaturated, with a balance being achieved between the input of gases via the bubbles and the evasion of gas through the sea surface. (Here is another feature of the asymmetry of the air-sea interface.) The bubble production increases with wind speed and so does the level of supersaturation at which equilibrium occurs. Present measurements suggest that in typical wave conditions, a mean wind speed of $\sim 8 \text{ m s}^{-1}$ may be sufficient to support a 3% supersaturation level in the water, about the average reported for oxygen in the Pacific⁴⁰. However, as M_v (and hence bubble area) increases rapidly with wind speed, an occasional storm may be sufficient to maintain enhanced levels of supersaturation equivalent to those produced by other processes (for example, biological).

Conclusion

We are slowly approaching improved, better determined and less empirical models of the upper ocean boundary layer through attention to small-scale processes. To be properly interpreted, these processes demand an understanding of the physics from scales upwards of a few micrometres, the size of the smallest bubbles. The processes are essential elements in the exchanges between the atmosphere and the ocean, and advances in understanding them will lead to improved estimates of the global budgets of energy, heat and gases.

Mr Alan Hall provided the data shown in Fig. 2.

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Solar signature in sedimentary cycles from the late Precambrian Elatina Formation, Australia

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Climatic cyclicity is recorded by regular variations in the thickness of varves in the Elatina Formation, a late Precambrian periglacial lake deposit in South Australia. Earlier conclusions that the climatic cycles reflect solar variability are strongly supported by a complexity of periods revealed through study of drill cores of the varved sequence. The new data promise to contribute significantly to our understanding of solar processes.

APPARENT solar periods (principally ~ 11 and ~ 22 yr) were previously recognized^{1,2} in thickness variations of varved (annually layered) periglacial lake deposits of the late Precambrian Elatina Formation in the Flinders Ranges, South Australia. Because the rocks are not well exposed, diamond drilling was carried out in December 1982 to obtain a complete cored sequence of the ~ 10 -m-thick varved interval. Some drill core was lost through rock fracturing, so a group of three vertical holes was drilled to allow matching of cores and construction of the longest possible continuous varved sequence. Logging the drill cores and correlation of the three sequences³ produced a stratigraphically continuous log, 9.39 m long and spanning an estimated $\sim 19,000$ yr VT (varve time), which could be measured precisely.

The new data reviewed here support the original conclusion that solar periods are recorded by the rocks, and may prove invaluable to our understanding of the solar activity cycle.

Geological setting

The Elatina Formation at Pichi Richi Pass in the Flinders Ranges, South Australia, comprises siltstones and fine sandstones deposited in a periglacial lake during the Marinoan Glaciation ~ 680 Myr ago¹⁻⁴. The occurrence of sand wedge structures in a penecontemporaneous fossil permafrost horizon within the adjacent palaeo-catchment area provides good evidence of a markedly seasonal, arid Marinoan periglacial climate⁴ and implies strong seasonal control on meltwater discharge into the periglacial lake.

The laminated interval of the Elatina Formation records climatic cyclicity through regular variations in the thickness and grain size of graded (fining-upward) laminae up to 3 mm thick (Fig. 1) that were deposited by density underflows in the distal portion of the lake^{1,2}. Scattered 'drop grains' up to the size of small pebbles were probably derived from melting river ice. The interval is characterized by conspicuous groups or cycles of about 10–14 laminae that are usually bounded by thinner, darker, more clayey laminae. The thickness of laminae varies systematically within each cycle, attaining a maximum near the cycle centre. A cycle 'doublet' of alternate thick and thin cycles is common (Fig. 1) and longer rhythms are evident through regular changes in cycle thickness.

The non-random variation in thickness of the graded laminae, together with the envisaged palaeogeographical setting and strongly seasonal arid palaeoclimate, provide good evidence that the laminae record non-random spring or summer meltwater discharge into the lake and that each lamina is, therefore, an annual increment or varve. The laminae are similar to the 'intermediate distal facies' in the varve classification of Smith⁵, which comprises delicate but distinct varves with summer layers usually too thin to display sublaminae. Through comparison with modern glaciolacustrine sedimentation⁶, varve thickness is interpreted as a measure of relative mean annual temperature. Plots

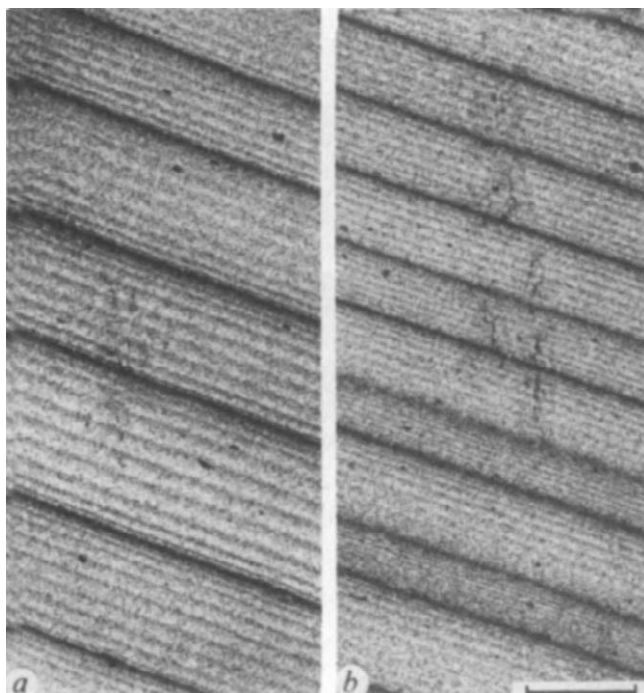


Fig. 1 Cyclic lamination in drill core from the Elatina Formation, Pichi Richi Pass. Conspicuous cycles comprise groups of ~ 10 –14 graded laminae of silt to very fine sand grade, bounded by thinner, darker, more clayey laminae. The lower half of *b* displays cycle 'doublets' resulting from alternate thick and thin successive cycles.

Scale bar, 1 cm.

of varve and varve-cycle thickness against time^{1,2} therefore may also be viewed as plots of time-variation in relative mean annual temperature. As the thinnest varves reflect the coldest portion of the climatic spectrum, their more clayey composition may be attributed to deposition of suspended fines through more widespread freezing or major overturn of cold surface waters¹. The scattered distribution of 'drop grains' throughout the cycles, with no preferred concentration in particular laminae, suggests, nonetheless, that significant pauses in annual density underflow events did not occur near varve-thickness minima.

Time-series analysis

From thickness measurements of drill-core material, distinct periodicities have been identified in two related sequences: (1) a detailed sequence of 1,337 varves obtained from enlarged photographs of thin-sections; (2) a long sequence of 1,580 cycles, each cycle comprising from 8 to 16 varves, obtained from the composite log of the drill cores.

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Table 1 Data for conspicuous cyclicity in the Elatina time series**a** Defined by varve thickness

	Mean period (yr VT)	s.d. (yr VT)	Range (yr VT)	Repetitions
Measured between minima	12.0	1.6	8–16	110
Measured between maxima	12.0	1.8	8–16	110

b Defined by maxima in ~12-yr VT cycle thickness ('Elatina cycle')

	Mean period (~12-yr VT cycles)	s.d.	Range	Repetitions
Unsmoothed curve (Fig. 3a)	26.2	1.5	20–29	59
Smoothed curve (Fig. 3b)	26.2	0.9	23–28	59

Table 2 Main periods identified in the Elatina time series

Visual mean period	DFT Period	PSD*	Visual mean period	DFT Period	PSD*
Detailed sequence (yr VT)			Long sequence (~12-yr VT cycles)		
	8.0	0.03		2.1	0.51
	9.7	0.06		3.8	0.01†
	10.3	0.29		4.4	0.02†
	11.0	0.46		5.3	0.04
	11.2	0.60		6.6	0.07
	11.6	0.23		8.8	0.16
12.0	11.9	0.66	13.1	13.1	0.55
	12.3	0.54	26.2	26.1	1.00
	12.5	0.64			
	13.0	0.30	Long sequence, low frequency ³ (~12-yr VT cycles)		
	13.5	0.24			
	14.4	0.16		134	0.21
	22.4	0.18		195	0.19
	25.8	0.21	700–800	778	1.00
	103.2	0.15			
~156	158.6	0.45			
~312	333.2	1.00			

* Power spectral densities (PSD) normalized to unity for the strongest periods.

† PSD from DFT of 'high-frequency' curve (see Fig. 3c).

Thicknesses were determined with a precision of 0.01 mm at the Laboratory of Tree-Ring Research and recorded on a floppy disk; the data were then transferred to the Lunar and Planetary Laboratory's INTERDATA 8/32 computer for time-series analysis.

Detailed sequence. Well-preserved varves contained in a piece of core 93 cm long were measured from photographic enlargements (4.4×) of overlapping thin sections³. In places very thin varves, possible random (intra-annual) layers¹, and rare soft-sediment deformation made counting uncertain. Such places were few, however, and indicate an accuracy of ±5% for the count of 1,337 measurements.

The 1,337 varves span 110 complete cycles near the top of the core sequence of 1,580 cycles (cycles 1,405–1,514). Conspicuous fluctuations in varve thickness (Fig. 2) define a cycle ranging from 8 to 16 yr VT in duration and with a mean period of 12.0 yr VT (Table 1a). The cycles show an overall tendency to positive skewness, the mean ascent period being 5.57 yr VT and the mean descent period 6.45 yr VT. Characteristically, varve thicknesses fall to approximately the same minimum level every ~12 yr VT (mean = 0.19 mm, s.d. = 0.07 mm), whereas the intervening maxima have a large range in thickness (mean = 1.20 mm, s.d. = 0.47 mm). Other features include the occurrence of multiple peaks for certain maxima (Fig. 2a), the very minor 'spikes' within some ~12-yr VT minima (Fig. 2a, b), and the common

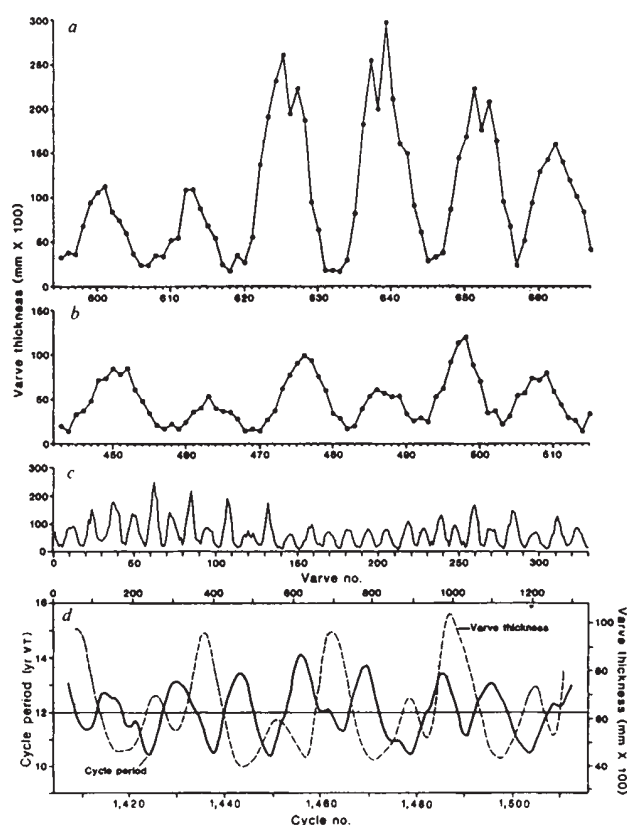


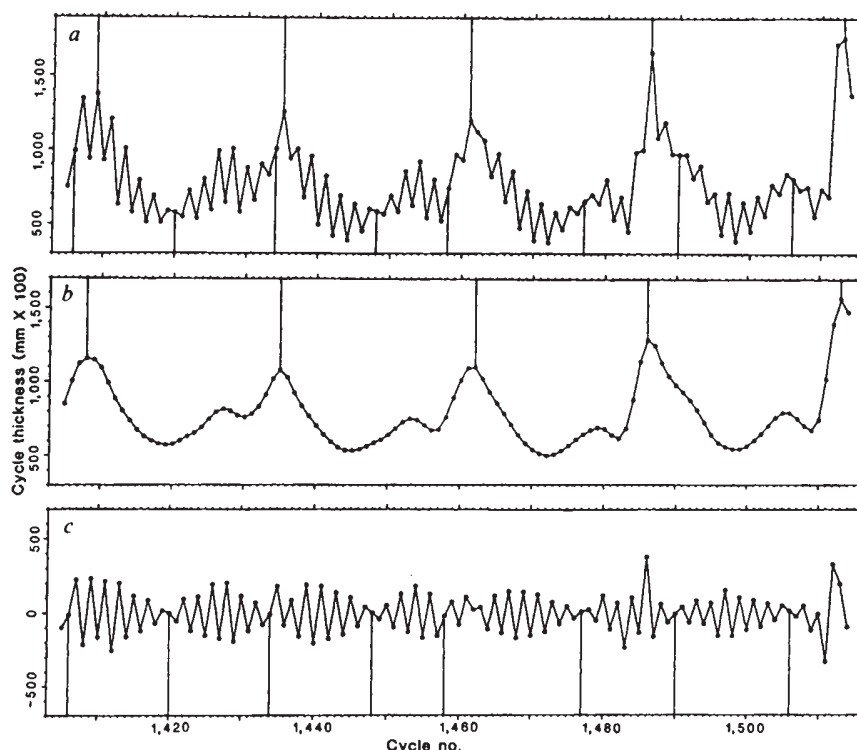
Fig. 2 Thicknesses of varves, extracted from the detailed sequence. (True thicknesses shown, obtained by dividing measurements from photographic images by 4.4.) Varve number increases up-sequence. **a**, Thick varves producing high-amplitude cycles with multiple peaks. A minor 'spike' occurs within a thickness minimum at varve 620. **b**, Thin varves producing low-amplitude cycles that display alternate higher and lower maxima. **c**, Small-scale plot showing long-term modulation of varve-cycle amplitude. **d**, Varve thickness for the entire detailed sequence (smoothed by 111-point filter), and variation with time in the period of the ~12-yr VT cycle measured between thickness minima (smoothed by five-point filter) for the same stratigraphical interval.

occurrence of alternating cycles of high and low maxima (Fig. 2b, c). In addition, regular fluctuations in the amplitude of the ~12-yr VT cycles and in varve thickness define longer periods of up to ~26 cycles (Fig. 2c, d).

The smoothed plot of cycle period against time (Fig. 2d) indicates that the long-term variation in cycle period is non-random; cycle periods are modulated by a longer rhythm with a mean period of about 13 cycles. Figure 2d shows that the thickest varves tend to coincide with relatively short cycle periods. Such negative correlation means that stronger ('higher') varve cycles tend to be of shorter duration than average. (This explains the early estimate of ~11 yr VT for the mean cycle period^{1,2}, as outcrop specimens displaying thicker, more easily counted varves and cycles were selected for examination first.) **Long sequence.** The excellent correlations obtained among the three drill holes indicate that a high level of confidence can be placed in the composite log of 1,580 ~12-yr VT cycles. Figure 3 shows the plot of cycle thickness against time for the interval of 110 cycles covered by the varve-thickness measurements, which is representative of the full sequence of 1,580 cycles.

Maxima in ~12-yr VT-cycle thicknesses occur on average every 26.2 cycles or ~314 yr VT (Table 1b). This long cycle, termed here the 'Elatina cycle', is repeated 59 times in the measured sequence. Second-order peaks that occur consistently in the same position relative to the maxima of the Elatina cycle

Fig. 3 Thicknesses of 110 ~12-yr VT cycles, extracted from the long sequence. Cycle number increases up-sequence. *a*, Cycle thicknesses showing distinct maxima every 25–27 cycles and a sawtooth pattern reflecting the characteristic alternation of relatively thick and thin cycles. *b*, Five-point smooth of the curve shown in *a*. *c*, High-frequency curve obtained by subtracting curve *b* from curve *a*. The vertical lines above the curves (*a*, *b*) mark boundaries of Elatina cycles and below the curves (*a*, *c*) positions of 180° phase reversals.



(Fig. 3b) confirm the presence of a second harmonic^{1,2} with a mean period near 13.1 ~12-yr VT cycles (~157 yr VT).

Superimposed on the Elatina cycle is a sawtooth pattern resulting from the alternation of relatively thick and thin ~12-yr VT cycles (Fig. 1b). The high frequency curve (Fig. 3c) reveals that the sawtooth pattern comprises a succession of envelopes displaying 180° phase reversals at or near the 'necks' between envelopes. A total of 106 such phase reversals occurs in the measured sequence; the interval between reversals averages 14.6 ~12-yr VT cycles (s.d. = 2.2, range = 9–23). The phase reversal is evidently a quasi-periodic function and no long-term modulation of the interval between reversals is apparent. The overall sawtooth pattern with phase reversals is described mathematically as a beat between two oscillations with periods near two cycles. As the mean period for a 360° change of phase (29.2 ~12-yr VT cycles or ~350 yr VT) is longer than the mean period of the Elatina cycle (26.2 cycles), the positions of phase reversals gradually advance up-sequence relative to amplitude maxima. The structure of amplitude maxima, therefore, varies according to their position relative to that of envelopes and necks of the sawtooth curve (Fig. 3a). As any miscount would break the observed pattern of phase reversals, the persistence of the pattern confirms the accuracy of the long sequence.

The Elatina Formation also records very long periods, notably a low-amplitude oscillation of period roughly 700–800 ~12-yr VT cycles discernible in the full plot of ~12-yr VT cycle thicknesses³.

Spectral analysis. The time series discussed above were analysed in several complementary ways: discrete Fourier transform (DFT), maximum entropy and least squares. All three methods gave similar results with regard to periods identified, although only the DFT results are discussed here. The periods determined by spectral analysis confirm those previously identified visually (Table 2), and are divisible into periods <30 yr VT, the Elatina cycle and its harmonics, and very long periods.

The DFT spectrum of the 1,337 varve-thickness measurements (Fig. 4a) reveals several discrete periods in the range of 10–14 and 22–25 yr VT, as well as longer periods of ~103, ~158 and ~333 yr VT. Additional, weaker peaks not shown in Fig. 4a occur at 8.0 and 9.7 yr VT. A beat between the periods identified at 22.4 and 25.8 yr VT may account for the beat pattern in the long sequence (Fig. 3c).

The DFT spectrum of the 1,580 ~12-yr VT cycles (Fig. 4b) reveals a striking 'harmonic series' comprising a fundamental period of 26.1 cycles and progressively higher harmonics of 13.1, 8.8, 6.6 and 5.3 cycles. Spectral plots at more detailed scales than Fig. 4b reveal two further harmonics at 4.4 and 3.8 cycles. The strong period near 2 cycles represents the ubiquitous cycle doublet. Good agreement exists between mutual periods identified in the detailed and long sequences, although the period of 26.1 cycles (Fig. 4b) is better resolved than the equivalent period of ~333 yr VT (Fig. 4a), which is beyond the limit of accurate resolution for the detailed sequence. Indeed, the consistency between data for the two sequences argues for the overall accuracy of the detailed sequence.

The Elatina cycle comprises, in accord with Fourier series expansion, a fundamental frequency and a number of higher harmonics (in the present case, six) of that fundamental. The DFT power spectral density amplitudes (*A*) of the Fourier series coefficients define an exponential function which is approximated by the equation

$$A \approx 220 \exp(-0.8f)$$

where *f* is the harmonic number (C. J. Durrant, personal communication). The harmonic series is also represented in the spectra of the detailed sequence (Fig. 4a).

DFT of the low-frequency portion of the long sequence suggests very long periods around 134, 195 and 778 ~12-yr VT cycles (~1,600, ~2,340 and ~9,330 yr VT respectively). The longest period is consistent with the low-amplitude oscillation discernible in the data³ but, statistically, is of uncertain significance in view of the relatively short record analysed.

As noted earlier, the period of the ~12-yr VT cycle appears to be modulated by a period near 13 cycles (Fig. 2d). DFT of cycle period versus time indicates that the period of the ~12-yr VT cycle is modulated principally by a period of 13.6 ~12-yr VT cycles (relative power spectral density = 1.00) and to a lesser degree by periods of 2.2 (0.73), 3.7 (0.18) and 27.8 (0.18) cycles (the latter period is, however, beyond the limit of accurate resolution). The periods of about 27.8 and 13.6 ~12-yr VT cycles may be equated respectively with the fundamental and second harmonic of the 'harmonic series' in the amplitude spectrum. Although the fundamental harmonic predominates in modulating ~12-yr VT-cycle amplitude, it is the second harmonic that

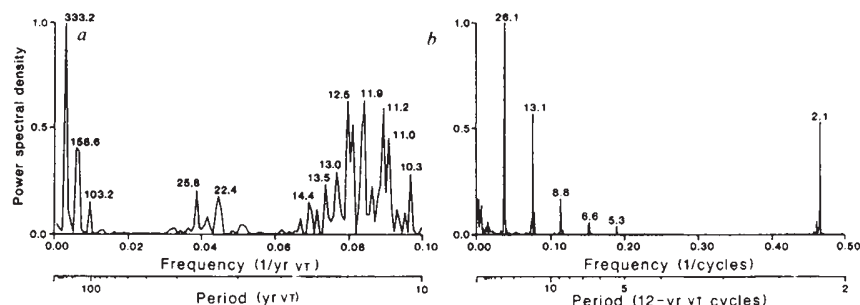


Fig. 4 DFT spectra normalized to unity for the strongest lines. *a*, DFT of 1,337 varve-thickness measurements. Spectrum unsmoothed. Linear frequency scale. *b*, DFT of thickness measurements of 1,580 ~12-yr VT cycles. Spectrum unsmoothed. Linear frequency scale. Peaks for the first to fifth harmonic of the (26 × ~12-yr VT) Elatina cycle are discernible.

predominates in modulating cycle frequency. The period near 2 cycles reflects a tendency for cycles of short duration to alternate with cycles of long duration, although such alternation in period shows no systematic relation to the amplitude alternation of the ~12-yr VT cycle (Fig. 3c).

Discussion

The periods recorded by the Elatina Formation cannot be explained by endogenetic processes within a glaciolacustrine system¹. Similar cyclicity has now been observed in penecontemporaneous lacustrine deposits up to 400 km from Pichi Richi Pass, indicating widespread climatic control of sedimentation. The new data generated by study of the drill cores support earlier conclusions^{1,2}, based on the study of limited outcrop, that the recorded climatic periods ultimately reflect solar variability.

The Elatina periods of 10–14, 22–25 and ~105 yr VT may be equated with cycles in the sunspot record, which similarly comprise several discrete periods between 8 and 13 yr, the Hale cycle of ~22 yr, and a longer period between 90 and 110 yr (refs 7–9). The longer Elatina periods of ~157 and ~314 yr VT are comparable to solar and climatic periods for Holocene time as indicated by tree-ring studies^{2,10,11}. The very long periods of up to ~9,300 yr VT may reflect very long-term climatic oscillations; possible counterparts exist in the recent climatic record^{12,13}, although a solar connection for such periods is not established.

The plot of varve thickness against time (Fig. 2) is comparable to that of mean annual sunspot number since AD 1700, particularly the reliable record since about AD 1830. Cycles in both the varve and sunspot series display a wide range of periods (8–16 yr VT for the Elatina Formation, 9.0–13.6 yr between minima and 7.3–17.1 yr between maxima for sunspot cycles¹⁴). The structure of cycles in each series is also comparable: minima fall to approximately the same level whereas maxima display a wide range of values; alternating cycles of high and low maxima are common; and varve cycles display an overall tendency to positive skewness, a feature also displayed by sunspot cycles^{15,16}. Furthermore, some varve-cycle maxima have multiple peaks, and minor 'spikes' occur within some minima; comparable features are displayed by the plot of mean annual sunspot (umbral) areas since AD 1920 (ref. 15). The similarity of structure of varve and solar cycles argues for a direct connection between varve thickness and solar activity, a conclusion reached previously on the evidence of overall skewness of a limited sample of varve cycles and the close similarity of portions of the varve-

cycle thickness curve to the plot of annual mean sunspot number at maxima^{1,2}. Varve-cycle structure would be quite dissimilar for an inverse relation between varve thickness and sunspot number.

The frequency modulation of the ~12-yr VT cycle (Fig. 2d) militates against the graded laminae representing monthly tidal increments, as such modulation is incompatible with regular tidal periods; indeed, the Elatina periodicities afford no evidence of tidal influence on sedimentation. Interestingly, the duration of the sunspot cycle over the reliable portion of the solar record since AD 1830 appears modulated by a longer period¹⁶, most minimum-to-minimum cycle lengths last century grouping around 11.5 yr and this century near 10–10.5 yr. A further similarity between the Elatina and solar records is the tendency for cycle duration to vary inversely with cycle amplitude. In the Elatina record, stronger ~12-yr VT cycles tend to be of shorter than average duration (Fig. 2d), and sunspot cycles of large amplitude tend to be of short duration, and vice versa¹⁷.

Portions of the plot of ~12-yr VT cycle thicknesses may be compared with the reliable sunspot record since AD 1830. Similarities include the structure of certain Elatina cycle (26 × ~12-yr VT) amplitude maxima and that of the solar maximum culminating in AD 1957, and the sawtooth pattern of cycle amplitudes (which maintains phase across the AD 1957 solar maximum). Such similarities between the Elatina and solar series are evident in Fig. 5. Cross-correlation between the overlapping portions of the curves shown in Fig. 5 yields the highly significant correlation coefficients (Pearson) of 0.95 (curves *a* and *b*) and 0.93 (curves *a* and *c*). That only the reliable portion of the sunspot record may be compared in this way with part of the Elatina curve increases confidence that the Elatina Formation indeed records solar variability.

Overall, the evidence from both the Elatina detailed and long sequences supports earlier conclusions^{1,2} of a direct connection between solar variability, climatic temperature change, and varve-thickness cyclicity during the Marinoan Glaciation in South Australia. In other words, increase in solar activity caused a corresponding increase in climatic temperature that resulted in greater annual meltwater discharge and the deposition of thicker varves. Importantly, recent varves deposited in glacial Skilak Lake in Alaska⁶ reveal a relatively weak solar signal recorded in this way¹⁸. Nonetheless, solar influence on climate must have been much greater during deposition of the Elatina Formation than is evident now. The composition of the late Precambrian atmosphere¹⁹ probably differed greatly from that

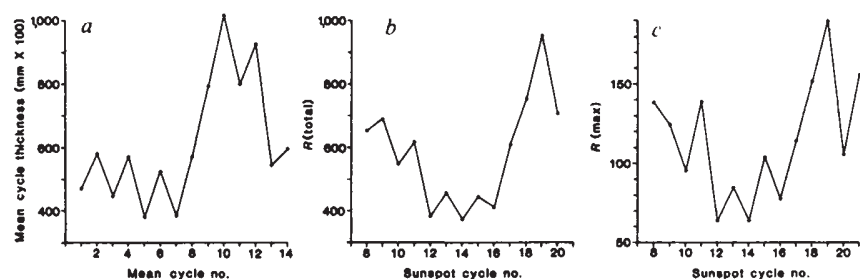


Fig. 5 Comparison of amplitude curves for ~12-yr VT cycles and sunspot cycles. *a*, Mean of five amplitude peaks from the Elatina long sequence (centred around cycles 89, 176, 855, 1,196 and 1,250) across which the phase of the sawtooth pattern is maintained. (A 'mean peak' was determined to reduce random noise in the geological data.) *b*, Total annual mean sunspot number for sunspot cycles since AD 1830. *c*, Annual mean sunspot number *R* at maxima of sunspot cycles since AD 1830.

of today, however, and the global climate may then have been much more sensitive to solar variability than is now observed³.

The Elatina record, in view of its clarity and great length, accordingly may provide invaluable data on which to model the physics of the solar activity cycle^{3,17}. The implications of these new data for solar physics and planetary science will be addressed separately.

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Thermally induced residual topography within oceanic lithosphere

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The lithosphere, following a thermal event, should subside isostatically to its pre-event configuration. However, if the thermal event is sufficient to reset the rigidity of the lithosphere, then the subsidence is forced to follow a different path to the initial uplift; lithospheric rigidity decrease during uplift allows flexural deformation resetting, while during subsidence, an increasing rigidity with time results in flexural interference. This 'thermo-mechanical hysteresis' effect may be responsible for anomalous bathymetry and free-air gravity anomalies associated with hotspot traces, extinct spreading centres and the ocean/continent boundary of rifted (passive continental) margins.

BROAD-SCALE reheating of the lithosphere is the result of thermal processes such as hotspot volcanism, rifting and possibly small-scale mantle convection. Concomitant with thermal modification of the lithosphere is its isostatic uplift and mechanical weakening (rigidity decrease). The actual uplift topography is a function of only the minimum rigidity obtained during heating. Subsequent cooling of the lithosphere allows its subsidence and mechanical recovery (increasing flexural rigidity with time). However, in marked contrast to the uplift, the cumulative subsidence is a function of the entire mechanical recovery history of the lithosphere. Flexural interference between this cumulative uplift and the initial topography produces a residual topography, the amplitude and wavelength of which critically depends on the rate of mechanical recovery and the degree of lithospheric heating.

The production of residual topography, as it involves the entire crust, resembles a buckled elastic plate. Even though such topography will necessarily be associated with large free-air gravity anomalies, the forces responsible for the deformation remain in mechanical and isostatic equilibrium at all times as evidenced by the equal positive and negative components of both the topography and free-air gravity anomaly. Formation of residual topography by the flexural interference of thermally modified lithosphere is here termed 'thermo-mechanical hysteresis' (TMH). It is suggested that TMH may be responsible for the characteristic free-air gravity anomalies observed over extinct spreading centres, for example, within the South China Sea and the Coral Sea basins, the isostatic gravity anomaly observed across the ocean/continent boundary of many passive margins, for example Argentina and the northeastern US margins, and anomalous bathymetric trends within the major

oceanic plates, for example, the linear bathymetric trend associated with the Cocos-Keeling Islands in the Indian Ocean.

Structure of the lithosphere

The interdependence of the thermal structure of the lithosphere and its mechanical properties has been convincingly demonstrated by recent studies of loading within the oceans and continents¹⁻⁵. Furthermore, these studies have established that flexure is the predominant mechanism by which emplaced loads are supported by the lithosphere. The form of the support is dependent on the lithospheric rigidity, which itself increases with lithospheric age. Generally, in the oceans, the plate age defines the thermal structure of the lithosphere through the cooling plate model⁶, while in the continents, rather than absolute plate age being important, it is the time since the last major thermal event as defined by either reset radiometric basement ages¹ or episodes of extensive alkalic or basic volcanism⁷.

Observations of the subsidence of seamounts associated with intraplate swells suggest that the swells behave like oceanic crust reset to a younger thermal age^{2,8-11}. The swells, in turn, are related to the broad-scale heating and hence thermal expansion of the lithosphere above hotspots (for example, Hawaii, Snake River province) or hot-lines (for example, Easter Island chain¹², eastern Australia^{13,14}). Thermal rejuvenation results in a lithosphere with properties identical to that of normal lithosphere but with an apparent younger thermal age and correspondingly lower lithospheric rigidity².

Thermal modification of the lithosphere by a general heating event causes an immediate isostatic uplift in an attempt to compensate the thermally expanded lower lithosphere^{15,16}. Isostatic compensation is essentially supplied by a Pratt-type pro-

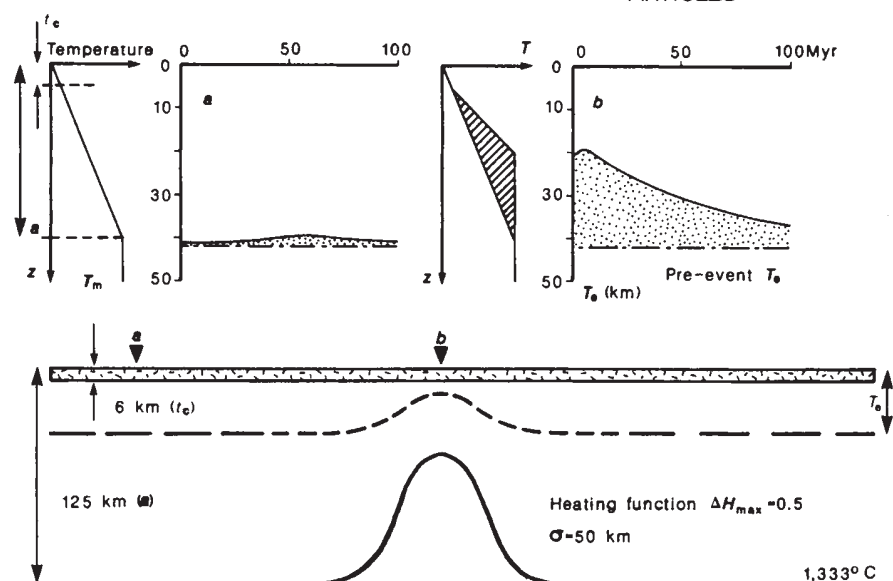


Fig. 1 Schematic representation of the initial rigidity structure of the lithosphere, the distribution of the heating function (parametrized by $\Delta H_{\max}$ and σ), the initial thermal structure over the zone of maximum heating and in the distant regions of the plate, and the mechanical recovery of the lithosphere (that is, the variation of T_e , the effective elastic thickness of the lithosphere) with time.

cess in which the uplifted topography compensates the lower densities associated with the thermally modified lithosphere. Even though Pratt isostasy is a local compensation scheme, the form and maximum amplitude of the surface deformation will be determined by the minimum lithospheric rigidity obtained during the thermal resetting process (in both space and time). Because plate rigidity is defined by the depth to a particular isotherm (in this case, the 450 °C isotherm⁵), minimum rigidity resetting occurs some time after the initiation of cooling as heat is removed vertically from the lower lithosphere to the upper lithosphere (Fig. 1). Second, minor heating associated with lateral heat flow eventually modifies rigidities in the distant regions of the plate. Following the maximum deformation stage, cooling of the lithosphere by vertical and lateral heat flow will result in a spatially and temporally varying flexural rigidity. In particular, the temporally increasing flexural rigidity will produce a progressively broader subsidence with time relative to the initial uplift. Flexural interference between this initial uplift and subsequent broader subsidence results in a residual topography. The purpose of this article therefore, is to model quantitatively the thermo-mechanical response of the lithosphere due to thermal loading and to define the production and form of residual topography.

Thermal loading

Several studies have suggested that mid-plate swells are adequately approximated by a gaussian-type heating function¹⁰. For convenience, the same functional form will be assumed for the distribution of the thermal load in this study. Heat input into the lithosphere can be characterized by the dimensionless quantity¹⁶ $\Delta H'$ where $\Delta H = 1$ represents complete melting of the lithosphere while $\Delta H = 0$ represents stable thermal equilibrium within the lithosphere. The degree of lithospheric heating can also be expressed in terms of the lithospheric stretching function of McKenzie¹⁵, and, in particular, the two-layer stretching model of Royden and Keen¹⁶, even though no literal stretching is implied. In particular,

$$\Delta H = (1 - 1/\beta) \left(\frac{a - t_c}{a} \right)^2 \exp[-x^2/\sigma^2]$$

$$\Delta H_{\max} = 0.5, \quad \beta_{\max} = 2.5$$

where $\Delta H_{\max}$ is the maximal thermal heating, β is typically the stretching of the sub-crustal lithosphere, σ is the half-width of the heating function (with modelling constants defined in Table 1), a is the lithospheric thickness, t_c the oceanic crustal thickness and x is the horizontal coordinate. As the lithosphere/asthenosphere boundary represents an isotherm (T_m), β can define not only the degree of sub-crustal stretching, but also the maximum

vertical migration of this temperature boundary and therefore the degree of subcrustal heating. The use of β does not necessarily imply a tectonic process or setting. However, the actual details of the heating process or the distribution of the thermal anomaly itself are not critical to this argument. Whenever a load, thermal or otherwise, is removed incrementally from the lithosphere while the flexural rigidity is varied, a permanent lithospheric deformation results. This article links the thermal loading (and subsequent unloading as the load is conductively dissipated) of the lithosphere with its thermal and hence mechanical modification (and subsequent recovery). By calculating $\beta(x)$ from ΔH , the initial (or instantaneous) surface uplift is¹⁷,

$$S_i = \frac{\alpha \rho_m T_m}{2(\rho'_m - \rho_w)} \Delta H(x)$$

where α is the thermal expansion coefficient, and ρ_m , ρ'_m and ρ_w are the mantle density at 0 °C, the asthenosphere density and the water density, respectively.

The initial surface uplift S_i , assumes that the lithosphere has zero flexural strength (Pratt or local isostasy) and is termed the

Table 1 Modelling parameters used

Parameter value	Definition
$a = 125$ km	Lithospheric thickness
$t_c = 6$ km	Oceanic crustal thickness
$\kappa = 0.008$ cm ² s ⁻²	Thermal diffusivity
$\alpha = 3.4 \times 10^{-5}$ °C ⁻¹	Thermal expansion coefficient
$T_m = 1,333$ °C	Temperature defining lithosphere
$\tau = 62.8$ Myr	Thermal relaxation coefficient
$Z_e = 450$ °C	Controlling elastic isotherm
$\rho_w = 1.03$ g cm ⁻³	Water density
$\rho_s = 2.5$	Sediment density } ρ_i
$\rho_m = 3.33$	Mantle density at 0 °C
$\rho'_m = \rho_m(1 - \alpha T_m)$	
$= 3.179$	Asthenosphere density
$g = 982$ cm s ⁻²	Acceleration due to gravity
$\gamma = 6.673 \times 10^{-8}$ cm ³ g ⁻² s ⁻²	Gravitational constant
$\Delta H_{\max} = 0.5$	Maximum thermal heating
$\beta_{\max} = 2.5$	Equivalent sub-crustal stretching value
$\sigma = 50$ km	Standard deviation of heating function
$D = Et_c^3/12(1 - \nu^2)$	Lithospheric rigidity
T_e	Effective elastic thickness
$E = 6.5 \times 10^{11}$ dyn cm ⁻²	Relaxed Young's modulus
$\nu = 0.25$	Poisson's ratio

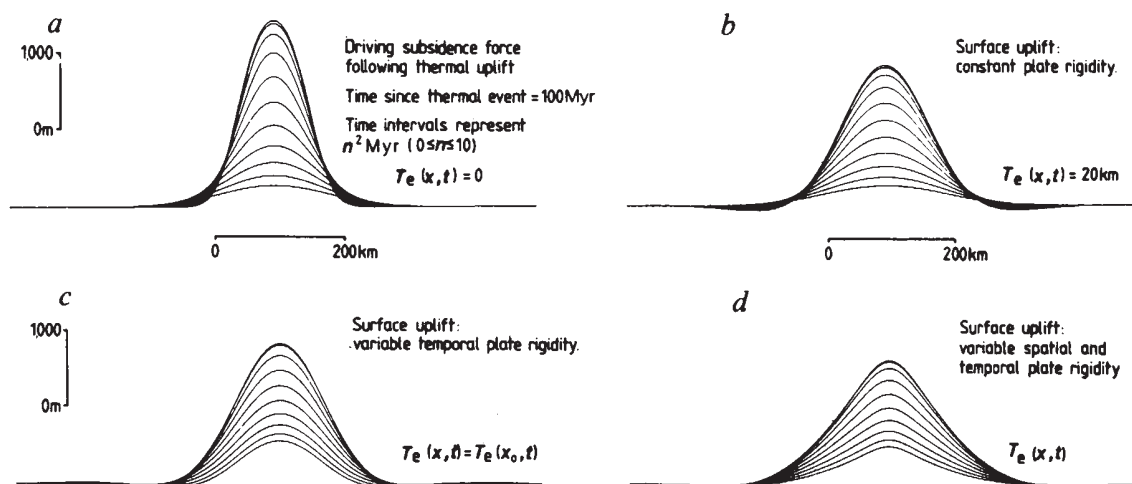


Fig. 2 Following a thermal event, the driving subsidence associated with the cooling and contraction of the lithosphere opposes the initial uplift, itself associated with a thermal buoyancy force. The resulting time-dependent force balance is subsequently modified by the mechanical properties of the lithosphere, producing a time-varying surface topography. The surface topography is illustrated for various assumptions of the mechanical response of the lithosphere at 0, 4, 9, 16, 25, 49, 64, 81 and 100 Myr since the initiation of cooling. Modelling parameters are defined in Table 1. *a*, Surface response of the lithosphere assuming a local isostatic response to loading. As local compensation refers to a zero-rigidity lithosphere, the form of the surface response is equivalent to the resultant force acting on the lithosphere. As lithospheric cooling has been modelled using both vertical and lateral heat flow, the force spreads laterally with time. *b*, Surface response of the lithosphere assuming a constant rigidity. If the thermal event fails to reset the lithospheric rigidity, the uplift and subsidence histories are identical and therefore no residual topography is produced. *c*, Surface response of the lithosphere assuming that its rigidity is reset and subsequently increases with time following the thermal event. The differing uplift and subsidence histories of the lithosphere result in a residual topography, the amplitude and wavelength of which relates to the pre-event rigidity and the degree of resetting. *d*, Surface response of the lithosphere assuming both a spatial and temporal variation in rigidity. The spatial rigidity variations relate to the distribution of the thermal event whereas the temporal variations relate to the thermal properties of the lithosphere. Note that in both *c* and *d* the topographic wavelength decreases with time, implying a progressive decrease in flexural rigidity. Such behaviour is usually interpreted in terms of the flexural response of a (Maxwell) visco-elastic plate to loading. However, in this case, the changing topographic wavelength is simply reflecting the sensitive interrelationship between the mechanical properties of the lithosphere and its temperature structure.

'local uplift'. Although Pratt isostasy (or any local isostatic scheme) generally represents an unrealistic response of the lithosphere to loading, it is very useful in describing the potential uplift of the lithosphere. Convolution of this local uplift with a flexural filter redistributes the surface uplift according to the mechanical properties of the lithosphere. For example, if the lithosphere has constant elastic rigidity D , then the surface uplift is given by (in the frequency domain)¹⁸,

$$S_i^* = \frac{\alpha \rho_m T_m}{2(\rho'_m - \rho_w)} \Delta H(k) \left[1 + \frac{Dk^4}{(\rho_m - \rho_i)g} \right]^{-1}$$

where ρ_i is the density of material infilling the deformation, k is the wavenumber, and g is the acceleration due to gravity. As a consequence of thermally modifying the temperature structure of the lithosphere, D is not constant but varies both spatially and temporally in the plate such that $D = D(x, t)$, t being the time since the initiation of lithospheric cooling.

Cooling of the lithosphere by the vertical and lateral flow of heat away from the thermally perturbed zone results in the creation of an opposing subsidence which progressively negates the initial surface uplift. The local subsidence counteracting S_i is given by¹⁹,

$$S_i(x, t) = \frac{2\rho_m \alpha T_m a}{(\rho'_m - \rho_m)} \sum_{n=1}^{\infty} \frac{2}{n^2 \pi^2} A_n^* (1 - e^{-n^2 t / \tau})$$

where

$$A_n = \frac{1}{n\pi} \{ (1 - \beta) \sin(n\pi z_1) + \beta \sin(n\pi z_2) \}$$

$$z_1 = t_c / a, \quad z_2 = z_1 + (1 - z_1) / \beta$$

$$A_n^* = A_n * X(x, t)$$

$$X(x, t) = \frac{1}{2(\pi \kappa t)^{1/2}} \exp[-x^2 / 4\kappa t]$$

where $X(x, t)$ represents the lateral conduction of heat, A_n the Fourier series of the initial vertical lithospheric temperature

distribution, $*$ the convolution operation, and τ is the thermal relaxation coefficient. As before, the actual subsidence is calculated by convolving $S_i(x, t)$, the local subsidence, with the lateral variations of lithospheric rigidity to give S_i^* . Figure 2a summarizes the spatial and temporal behaviour of the local thermal uplift (maximum value of 2,380 m) within the lithosphere for $\Delta H = 0.5$ ($\beta_{\max} = 2.5$) and $\sigma = 50$ km (and for times n^2 Myr since initiation of cooling, $1 < n < 10$) and clearly shows the effects of both vertical and lateral heat flow. Because the isostatic compensation in this example is assumed to be local, the uplift is proportional to the thermally generated buoyancy force acting on the lithosphere. With infinite time, the residual local uplift ($S_i - S_i(x, t)$), and therefore the buoyancy force, are identically zero everywhere.

Lithospheric subsidence

Referring to Fig. 2, we can monitor the subsidence of the lithosphere following a thermal event for a variety of mechanical conditions. If we insist on the mechanical homogeneity of the lithosphere both laterally and with time, the residual subsidence ($S_i^* - S_i^*(x, t)$) will again be reduced to zero given sufficient time even with flexural redistribution of the surface uplift (Fig. 2b). With $T_e = 20$ km and after 100 Myr, a topographic surface with amplitude 254 m is all that remains from an initial uplift of 1,824 m. If, however, we relax the requirement for temporal homogeneity of the lithospheric rigidity, then the flexural modification of the local subsidence changes continually with time. In this example, the pre-event effective elastic thickness of T_e (42.2 km) is thermally reset to 20 km. Mechanical recovery of the lithosphere over a 100-Myr period increases T_e from 20 to 36.9 km (Fig. 1), producing significant residual topography (431 m, Fig. 2c) relative to Fig. 2b.

Thermal modification of the lithospheric temperature structure will obviously be greatest nearest the thermal perturbation. Necessarily, therefore, the mechanical properties of the lithosphere must be a function not only of time since the initiation of cooling, but also of the distance from the perturbation. The

redistribution of the local subsidence, $S_i(x, t)$, by a heterogeneous elastic plate to give the flexural subsidence, $S_f^*(x, t)$ is given by,

$$\frac{\partial^2}{\partial x^2} \left[\frac{D(x, t)}{(\rho_m - \rho_i)g} \frac{\partial^2}{\partial x^2} S_f^*(x, t) \right] + S_f^*(x, t) = S_i(x, t)$$

The lateral and temporal variations of plate rigidity, $D(x, t)$, are calculated by monitoring the depth to the 450 °C lithospheric isotherm⁵. The above differential equation is most conveniently solved using a finite-difference algorithm²⁰. The resulting topography ($S_f^* - S_i^*(x, t)$) is now the result of a complex interaction between vertical and lateral heat flow and the spatial variations of plate rigidity about the thermal anomaly. Figure 2d represents the full model prediction for thermally reset oceanic lithosphere, producing in this example residual topography of 335 m. While the residual topography is only relatively small (100–200 m), note that this deformation was produced with only a modest degree of sub-crustal heating. Areas associated with high-temperature contrasts, such as during the creation of a passive continental margin or during the death of a mid-oceanic spreading centre, will enhance this hysteresis effect and, while probably never dominating, will always be a complicating factor in loading situations when heat is involved.

Apparent visco-elastic behaviour

A curious feature of the residual topography in Fig. 2c, d is that the wavelength appears to decrease progressively with time implying, therefore, an apparent decrease in flexural rigidity with time even though we are able to monitor its actual increase. That is, the surface deflection is mimicking the temporal response of a visco-elastic (Maxwell) plate. This observation simply underlines the sensitive interrelationship between lithospheric temperature structure and its flexural response and may prove to be useful in rationalizing the apparent contradiction posed by those flexural studies which vehemently refute the elastic model in favour of a visco-elastic (Maxwell) model^{21–26}.

Apparent visco-elastic behaviour of the lithosphere is not limited to TMH. McNutt²⁷, in her analysis of seamount flexure, noted that load rejuvenation occurs as the heat introduced into the lithosphere during seamount emplacement is vertically redistributed. The degree of load rejuvenation is therefore sensitive to the level at which the temperature anomaly is introduced. Similarly, foreland basins, produced as an isostatic response to thrust sheet/nappe emplacement²³, modify the temperature structure of the foreland lithosphere (at a rate determined by the thermal diffusivity of the lithosphere) as the overlying sediment infill and thrust sheets thermally re-equilibrate²⁸. In both cases, lithospheric heating induces a decrease in rigidity with time which mimics the behaviour of a Maxwell visco-elastic plate.

While thermo-mechanical hysteresis is concerned with load removal during cooling of the lithosphere, a similar flexural interference is obtained during (non-thermal) load emplacement. For example, Liu *et al.*²⁹ successfully modelled the negative gravity anomaly over the 85° E ridge by investigating the flexural interference between the basement deformation produced by the formation of the ridge and the regional deformation associated with its subsequent burial by sediments. Thermo-mechanical hysteresis, therefore, is only a special case of the flexural interference of lithospheric deformations associated with temporally and/or laterally varying loads and a temporally varying lithospheric rigidity.

Isostatic equilibrium

The isostatic state of the heated lithosphere will be a complicated interaction between the Pratt effect of the thermally modified subcrustal lithosphere and the regional isostatic effect of the evolving crustal deformation. In the limit, however, only the crustal deformation remains. Figure 3 illustrates the residual topography and associated free-air gravity effect produced by thermal re-equilibration of oceanic lithosphere over a 400-Myr period since cooling and for all practical purposes, represents

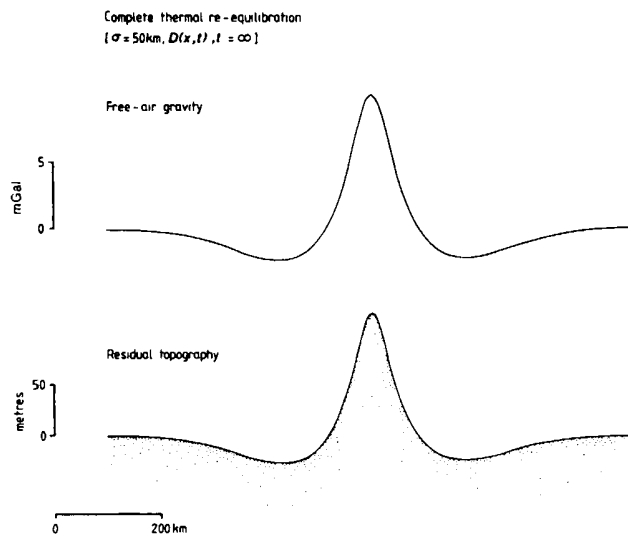


Fig. 3 The resultant free-air gravity effect and residual surface topography following thermal re-equilibration of the lithosphere ($t = 400$ Myr) in which the original thermal perturbation modified both the spatial and temporal flexural properties of the lithosphere. The residual topography and its free-air effect remain an isostatic process in the sense that the topography and free-air gravity laterally integrate to zero (remembering that the Moho parallels the surface).

complete thermal re-equilibrium of the lithosphere. If, during the thermal event, the lithospheric rigidity failed to be reset (that is, remains constant), no residual topography (and hence gravity effect) would be produced. In contrast, any rigidity variation (in space or time) results in a residual topography, in this example being 162 m with a corresponding free-air gravity effect of 20 mGal. Compensation is maintained by the strength of the lithosphere, not in the usual way, but by the production of a surface deformation which laterally integrates to zero (here termed mechanical equilibrium).

Applications

Thermo-mechanical hysteresis results from the thermal re-equilibration of the lithosphere following a heating event. This concept, when applied to the oceanic lithosphere in particular, may help to explain several anomalous bathymetric and gravimetric trends within the Indian Ocean (for example, Cocos-Keeling Islands and associated bathymetric trends), extinct spreading centres (for example, Coral Sea Basin) and ocean/continent boundaries of passive margins (for example, Argentinian margin). No attempt has been made to model the bathymetry or gravity anomalies in detail, but the similarity in characteristics predicted by thermo-mechanical hysteresis, namely, low-amplitude but long-wavelength topography, relatively large-amplitude free-air gravity anomalies relative to the topography (or equivalently, an anomalously high gravitational admittance) and an isostatic compensation reminiscent of mechanical isostasy (a balanced deformation of the entire crust generally reported as uncompensated topography), and the above geological features, strongly suggests that the same process, TMH, may be operative.

Cocos-Keeling hotspot trend

Anomalous north-east-south-west trending bathymetry (1–2 km) within the northern Indian Ocean and generally paralleling the absolute motion of the Indo-Australian plate (Fig. 4a) is associated with the Cocos-Keeling Islands, the trend and islands being produced by a presumed hotspot of Lower Tertiary age. Although the bathymetry of the trend is minor relative to the Ninety-East Ridge, the associated free-air gravity anomaly is excessively large (Fig. 4a). To remove the effect of the Cocos-

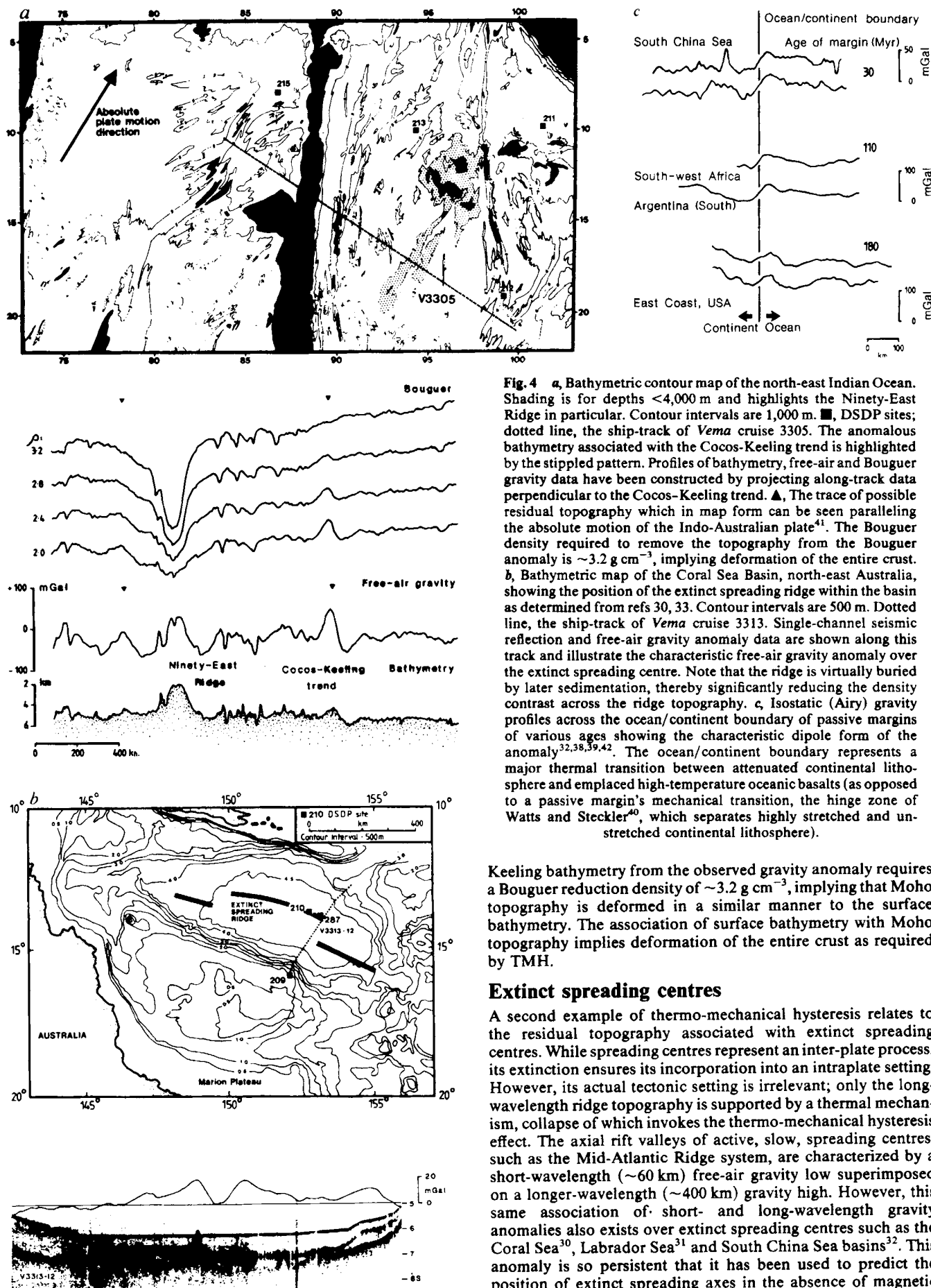


Fig. 4 *a*, Bathymetric contour map of the north-east Indian Ocean. Shading is for depths <4,000 m and highlights the Ninety-East Ridge in particular. Contour intervals are 1,000 m. ■, DSDP sites; dotted line, the ship-track of *Vema* cruise 3305. The anomalous bathymetry associated with the Cocos-Keeling trend is highlighted by the stippled pattern. Profiles of bathymetry, free-air and Bouguer gravity data have been constructed by projecting along-track data perpendicular to the Cocos-Keeling trend. ▲, The trace of possible residual topography which in map form can be seen paralleling the absolute motion of the Indo-Australian plate⁴¹. The Bouguer density required to remove the topography from the Bouguer anomaly is $\sim 3.2 \text{ g cm}^{-3}$, implying deformation of the entire crust. *b*, Bathymetric map of the Coral Sea Basin, north-east Australia, showing the position of the extinct spreading ridge within the basin as determined from refs 30, 33. Contour intervals are 500 m. Dotted line, the ship-track of *Vema* cruise 3313. Single-channel seismic reflection and free-air gravity anomaly data are shown along this track and illustrate the characteristic free-air gravity anomaly over the extinct spreading centre. Note that the ridge is virtually buried by later sedimentation, thereby significantly reducing the density contrast across the ridge topography. *c*, Isostatic (Airy) gravity profiles across the ocean/continent boundary of passive margins of various ages showing the characteristic dipole form of the anomaly^{32,38,39,42}. The ocean/continent boundary represents a major thermal transition between attenuated continental lithosphere and emplaced high-temperature oceanic basalts (as opposed to a passive margin's mechanical transition, the hinge zone of Watts and Steckler⁴⁰, which separates highly stretched and unstretched continental lithosphere).

Keeling bathymetry from the observed gravity anomaly requires a Bouguer reduction density of $\sim 3.2 \text{ g cm}^{-3}$, implying that Moho topography is deformed in a similar manner to the surface bathymetry. The association of surface bathymetry with Moho topography implies deformation of the entire crust as required by TMH.

Extinct spreading centres

A second example of thermo-mechanical hysteresis relates to the residual topography associated with extinct spreading centres. While spreading centres represent an inter-plate process, its extinction ensures its incorporation into an intraplate setting. However, its actual tectonic setting is irrelevant; only the long-wavelength ridge topography is supported by a thermal mechanism, collapse of which invokes the thermo-mechanical hysteresis effect. The axial rift valleys of active, slow, spreading centres, such as the Mid-Atlantic Ridge system, are characterized by a short-wavelength ($\sim 60 \text{ km}$) free-air gravity low superimposed on a longer-wavelength ($\sim 400 \text{ km}$) gravity high. However, this same association of short- and long-wavelength gravity anomalies also exists over extinct spreading centres such as the Coral Sea³⁰, Labrador Sea³¹ and South China Sea basins³². This anomaly is so persistent that it has been used to predict the position of extinct spreading axes in the absence of magnetic

anomalies^{32,33}. Once spreading has ceased within a rift axis, heat is rapidly dissipated, allowing the ridge topography to subside and thereby destroying its characteristic waveform. In general, the isostatic compensation of spreading ridge topography is poorly understood. Interpretation of admittance functions obtained across active spreading systems^{34,35} suggests low lithospheric strengths ($T_e < 10$ km). Seismic studies have shown, however, that oceanic crust is of normal thickness at least to within ~ 10 m of the ridge axis³⁶. As the crustal thickening predicted by isostatic models (in which ridge topography is represented as a surface load) is not observed, such models fail to describe adequately the isostatic state of the ridge. The most promising approach has been to model the ridge topography as the response of a broken elastic plate to a sublithospheric buoyancy force³⁷, analogous to the thermal event of Fig. 2a. The extinction of the spreading ridge system means the eventual dissipation of the thermally generated buoyancy force, ridge subsidence, and as the lithosphere mechanically recovers, the 'freezing-in' of the ridge topography by thermo-mechanical hysteresis.

The extinct spreading centre of the Coral Sea Basin (Fig. 4b) is associated with a short-wavelength (~ 50 km) free-air gravity anomaly low of 15–20 mGal and a longer-wavelength (~ 350 km) gravity high³⁰ (Fig. 4b). Since the rift valley topography has been buried by turbidites infilling the Coral Sea Basin from Australia and New Guinea, the source of the free-air gravity anomaly is approximately shared between the crust-sediment and crust-mantle interfaces. Calculating the gravity effect of each interface using the Bouguer approximation gives:

Interface (1): $42.2 \text{ mGal km}^{-1} \text{ g}^{-1} \text{ cm}^{-3}$

Upward continuation: $\exp(-kd)$; k , the wavenumber, is defined as $2\pi/L$, L being wavelength (~ 50 km) and, therefore, $k = 0.126$, and d , the mean depth of the interface, is ~ 5 km.

$$42.2(2.8 - 2.5)1.5 \exp(-0.126 \times 5) = 10 \text{ mGal}$$

Interface (2): $d = 10$ km, $k = 0.126$

$$42.2(3.33 - 2.8)1.5 \exp(-0.126 \times 10) = 9.5 \text{ mGal}$$

The combined free-air gravity effect is 19.5 mGal, indicating that the short-wavelength acoustic topography of the Coral Sea rift valley is conformable with the Moho topography. Given the accumulating evidence for the existence of an elastic layer supporting both active and inactive rifts³¹, it would appear that the 'frozen' ridge topography is most simply explained (though not explicitly modelled) in terms of the thermo-mechanical hysteresis of this elastic layer and is responsible for the observed crustal configuration and free-air gravity anomaly.

Ocean/continent boundaries

The final example relates to the persistent free-air gravity anomaly associated with ocean/continent boundaries, often highlighted by the calculation of an Airy isostatic anomaly, of many passive continental margins. The ocean/continent bound-

ary is characterized by a steep landward gradient, the midpoint of which identifies the position of the boundary as collaborated by magnetic anomalies and reflection seismics^{32,38,39}. Some margins also have an associated basement high which appears to be uncompensated³⁹. The form of the isostatic anomaly approximates a dipole, centred on the ocean/continent boundary, with the positive and negative components relating to the oceanic and continental sides respectively (Fig. 4c). A cause in terms of differential subsidence and flexure between the oceanic and continental crust is considered unlikely because, as noted by Watts and Steckler⁴⁰, the mechanical properties of thinned continental and oceanic lithosphere are similar and, therefore, it is not obvious why a flexural deformation between unstretched continental crust and thinned continental/oceanic crust should be centred on the ocean/continent boundary. In the case of the South China Sea (margin age ~ 30 Myr), the isostatic anomaly³² has an amplitude of ~ 30 mGal, ~ 400 km wavelength, and landward gradient $0.85 \text{ mGal km}^{-1}$ (Fig. 4c). In contrast, the significantly older Argentinian margin (age ~ 110 Myr) has an anomaly amplitude of ~ 60 mGal, 400 km wavelength, and gradient $0.95 \text{ mGal km}^{-1}$ (that is, the admittance tends to increase with increasing margin age). The steep gradient demands a relatively shallow source (crustal) while the wavelength and dipole nature of the anomaly suggests that compensation is related to the deformation of the crust. The association of this anomaly with a thermal process across the ocean/continent boundary (the initial continental rifting) again suggests that the ocean/continent boundary anomaly is a product of thermo-mechanical hysteresis.

Conclusions

Long after the cessation of inter- or intraplate thermal activity, the surface of the lithosphere will remain distorted with an amplitude and wavelength dependent on the mechanical properties of the lithosphere and the degree of reheating. This residual topography will remain indefinitely unless reset and hence redistributed by a subsequent thermal event. As the production of residual topography is a hysteresis process, the previous deformation is only replaced by a new deformation—the surface can never return to a stress-free state. Suitable thermal events include intraplate hotspots, formation of ridge topography at spreading centres, and continental (oceanic) rifting leading to the formation of an ocean/continent (ocean/ocean) boundary. Given the ability of the lithosphere to recover mechanically from a superimposed thermal event, the existence of TMH is an undeniable consequence. However, further work will be required to determine its exact importance.

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The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice

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Transgenic mice bearing the cellular myc oncogene coupled to the immunoglobulin μ or κ enhancer frequently develop a fatal lymphoma within a few months of birth. Since the tumours represent both immature and mature B lymphocytes, constitutive c-myc expression appears to be highly leukaemogenic at several stages of B-cell maturation. These myc mice should aid study of lymphoma development, B-cell ontogeny and immunoglobulin regulation.

THE arguments that a small number of cellular genes, the proto-oncogenes, are crucial to the development of neoplasia are persuasive^{1,2}. Nevertheless, the evidence that natural malignancies are caused by somatically altered proto-oncogenes has remained largely circumstantial³. Transgenic mice (reviewed in ref. 4) provide the means to test directly the efficacy of cellular oncogenes *in vivo*. Since a gene injected into a fertilized ovum typically integrates into a host chromosome within a few cell divisions, all tissues of the transgenic mouse usually acquire the gene, but the regulatory elements of the gene may direct tissue-specific expression⁴. Introduced immunoglobulin genes, for example, are expressed only in the lymphoid cells of transgenic mice⁵⁻⁸.

Recently, both a viral and a cellular oncogene have been shown to elicit characteristic tumours in transgenic mice. The type of tumour induced by the large-T antigen gene of simian virus 40 (SV40) depends on the element controlling its expression; with the SV40 enhancers, papillomas of the choroid plexus develop^{9,10}, while the insulin regulatory region leads to pancreatic tumours¹¹, and the metallothionein-I control region yields still other neoplasms¹². The cellular *myc* oncogene linked to the regulatory region within the long terminal repeat (LTR) of the mouse mammary tumour virus promotes mammary carcinomas¹³. These results suggest that tissue-specific regulators can target the action of oncogenes to particular cell types.

We wished to establish whether the *c-myc* gene can be introduced into the mouse genome in a form that promotes lymphoid malignancy. Altered regulation of *c-myc* expression has been strongly implicated in lymphoid neoplasia. In retrovirus-induced avian B lymphomas^{14,15} and some rodent T lymphomas^{16,17}, proviral insertion near *c-myc* brings it under the control of the promoter or enhancer within the retroviral LTR. In most human Burkitt's lymphomas and murine plasmacytomas, the *myc* gene has been activated by translocation to the immunoglobulin heavy (*IgH*)-chain locus (reviewed in refs 18-20). The way in which the *IgH* locus activates *c-myc* expression is poorly understood, but the lymphoid-specific *H* locus enhancer (E_{μ})²¹⁻²⁴ may well be the activating element in those translocations that couple it to *c-myc*.

By constructing transgenic mice bearing different forms of the *c-myc* gene, we have established that subjugation of this proto-oncogene to immunoglobulin enhancers converts it into a potent leukaemogenic agent for B lymphoid cells. Our results also support the notion that expression of the normal *myc* gene is subject to feedback regulation^{18,25} and bear upon the issue of whether an activated *c-myc* gene alone is sufficient to cause tumours. Since the predisposition is heritable, the novel lines of *myc* mice described here should prove valuable in unravelling early events in lymphoma development, in defining early stages

of lymphoid ontogeny, and in clarifying the mechanisms that regulate immunoglobulin gene expression.

Enhancers make c-myc tumorigenic

The seven constructs we have introduced into mice include an intact murine *c-myc* gene (top), a truncated form bearing the coding region (exons 2 and 3) and five versions with *c-myc* coupled to other regulatory regions (Fig. 1a). In the E_{μ} -*myc* construct, which is equivalent to the rearranged *c-myc* allele in the plasmacytoma ABPC17 (ref. 26), the *H* locus enhancer lies upstream of exon 1, while in the LTR-*myc* construct the enhancer region of a murine retroviral LTR is positioned similarly¹⁶. In E_{κ} -SV-*myc*, the lymphoid-specific immunoglobulin κ enhancer^{6,27,28} is coupled through the SV40 promoter to *myc* exons 2 and 3. SV-*myc* and MT-*myc* are analogous constructs containing the enhancer plus promoter of SV40 or the murine metallothionein-I gene, respectively. In four of the constructs the *myc* 3'-untranslated region was marked (Fig. 1b) with an irrelevant sequence (0.6 kilobases (kb) of Φ X174 phage DNA) to distinguish its transcripts from those of the endogenous *myc* gene.

To make transgenic *myc* mice, (C57BL $\times$ SJL)F₂ eggs were injected with a construct, implanted in pseudopregnant females, and transgenic pups identified by hybridization to tail DNA²⁹. Many of these primary transgenic animals developed tumours, and the incidence is expressed in Fig. 1a as a fraction of the mice bearing each construct.

Significantly, both the μ and κ enhancer constructs elicited tumours and all involved lymphoid tissue. The E_{μ} -*myc* gene was remarkably potent, 13 of 15 primary transgenic animals developing lymphomas (see below). These mice died, or became terminally ill (and hence were killed) between 6 and 15 weeks of age (median 11 weeks). With E_{κ} -SV-*myc*, 6 of 17 transgenic animals died with lymphomas between 11 and 44 weeks of age (median 23 weeks). The efficacy and specificity of these constructs are almost certainly due to the immunoglobulin enhancers, because no tumours have arisen during 10 months of observation in the mice carrying either the tagged normal *myc* gene or the truncated form typical of most murine plasmacytomas^{19,20}. Since both immunoglobulin constructs were effective in multiple primary transgenic animals, each representing an independent insertion event, these enhancers must function in diverse chromosomal environments, as can intact μ and κ genes⁵⁻⁸.

The non-immunoglobulin constructs gave few tumours. The LTR enhancer-*myc* construct, which is derived from a T lymphoma¹⁶, gave rise to only one tumour, a thymic lymphoma. With the SV-*myc* construct, which is equivalent to one that transforms very effectively *in vitro*³⁰, 3 of 21 mice developed tumours: a lymphosarcoma, a renal carcinoma and a fibrosar-

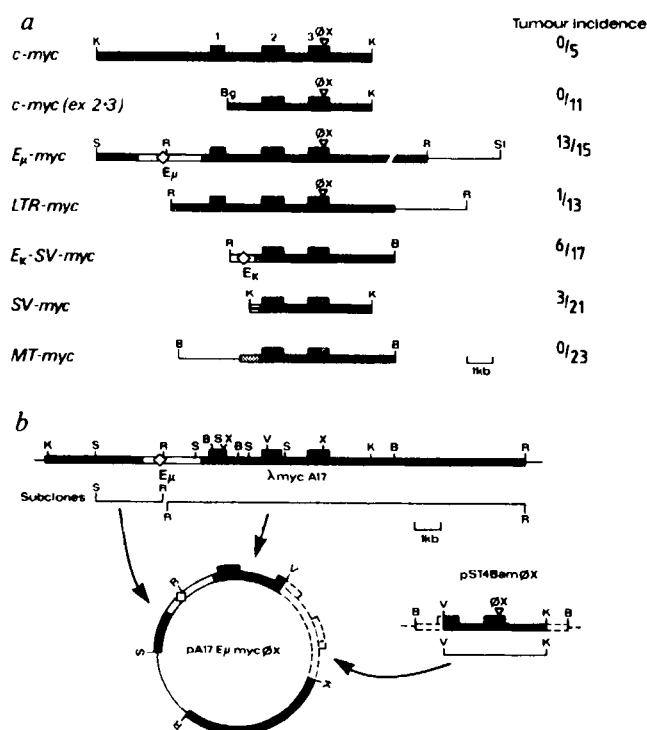


Fig. 1 **a**, The *c-myc* constructs injected into fertilized ova and tumour incidence in resulting transgenic mice. The solid bar denotes *c-myc* sequences with exons raised, untranslated regions striped, the ΦX174 marker DNA (see **b**) as a triangle, and vector sequences as a thin line. The linear fragments shown were isolated from plasmid constructs (described below) before injection. The *E_μ-myc* construct contains 2.3 kb of immunoglobulin DNA (open bar) spanning the H-chain enhancer (diamond), inserted 361 base pairs (bp) 5' to *c-myc* exon 1, as in plasmacytoma ABPC17 (see **b** and ref. 26) and extends 7.2 kb 3' to *c-myc*. The *LTR-myc* construct has the enhancer-containing 210 bp of the Soule murine leukaemia virus LTR (cross-hatched) inserted ~1.3 kb 5' to *c-myc*, as in T lymphoma ST4 (ref. 16). The bottom three constructs involve fusion to the *Xba*I site directly 5' to *c-myc* exon 2 (ref. 45). *E_κ-SV-myc* has an ~900-bp fragment of *κ* locus DNA (open bar) containing the *κ* enhancer (diamond) linked to the SV40 promoter (181-bp *Sph*I/*Stu*I fragment); *SV-myc* has the complete SV40 promoter/enhancer region (347-bp *Kpn*I/*Stu*I fragment); and *MT-myc* bears metallothionein-I sequences (stippled), from -750 to +6 (ref. 52), including its heavy metal-regulated promoter. **b**, Construction of *c-myc* plasmids. To mark the ABPC17 *c-myc* gene (ref. 26) within its 3'-untranslated region, the *Eco*RI fragment spanning *c-myc* was subcloned, and its *Eco*RV/*Kpn*I fragment exchanged for one bearing the ΦX174 marker. The latter was taken from pST4BamΦX, constructed by ligating a ΦX174 *Hae*III/*Xho*I fragment (nucleotides 4,984-162) via *Xho*I linkers to a *Xho*I-restricted *Bam*HI subclone isolated from the T lymphoma ST4 (ref. 16). Finally, the ΦX-marked *Eco*RI fragment was inserted at the *Eco*RI site of the ABPC17 *Sac*I/*Eco*RI subclone to give pA17E_μmycΦX. For injection, this plasmid was linearized with *Sall*, which cuts within the pUC12 vector. The *c-myc* (ex 2+3) fragment was excised from pA17E_μmycΦX with *Bgl*II and *Kpn*I. The marked normal *c-myc* construct (**a**, top) was prepared analogously by replacing the 5' segment of pST4BamΦX (up to the unique *Eco*RV site in exon 2) with an 11-kb *Eco*RI/*Eco*RV fragment spanning the 5' end of the unrearranged *c-myc* gene; the 11.2-kb *Kpn*I fragment was then excised for injection. The *LTR-myc* plasmid was constructed by inserting the ΦX marker into an ~14-kb rearranged *c-myc* fragment from ST4 (ref. 16) by *Eco*RV/*Kpn*I exchange with pST4BamΦX as above, and the isolated *Eco*RI fragment injected. The *E_κ-SV-myc* plasmid was constructed by replacing the SV40 enhancer (*Sph*I/*Kpn*I) with a 900-bp fragment (nucleotides 3,390-4,290 in ref. 53) containing the *κ* enhancer. For *MT-myc*, the *Xba*I/*Bam*HI fragment spanning *myc* exons 2 and 3 was inserted into a MT vector containing a *Xba*I linker at +6 and a 3' *Bam*HI site; it was linearized at *Bam*HI for injection. Restriction endonuclease sites are: K, *Kpn*I; Bg, *Bgl*II; S, *Sac*I; R, *Eco*RI; Sl, *Sall*; B, *Bam*HI; X, *Xho*I; V, *Eco*RV.

coma. This diversity echoes the wide range of cells in which the SV40 enhancer functions. The absence of tumours of the choroid plexus with this construct may mean that the *myc* gene product is less effective than the large-T antigen^{9,10} in transforming brain epithelial cells. We were surprised that no tumours ensued with the MT-*myc* construct, because that control region has induced expression of other genes in transgenic mice^{12,31,32}, but the vector sequences present in this construct (Fig. 1a) may have had a suppressive effect³³.

Aggressive lymphoma

The striking efficacy of the *E_μ-myc* construct led us to concentrate on mice bearing it. To establish whether the predisposition to lymphoma development was heritable, we analysed progeny from primary *E_μ-myc* mice that survived long enough to allow breeding. In eight independent lineages, lymphomas have developed with high fidelity. Indeed, in three generations of descendants, 59 of 63 mice (94%) have died by 4 months of age. The pathology is very similar in all lineages.

The hallmark of the disease in *E_μ-myc* mice is massive enlargement of the lymph nodes. The onset, most readily assessed by palpation of the inguinal lymph nodes, usually occurs between 3 and 18 weeks of age. A few weeks later, most mice develop 'water wings', prominent lumps on both shoulders and the neck (Fig. 2a), reflecting swelling of the brachial and cervical lymph nodes, and most other nodes are grossly enlarged (Fig. 2b). The spleen usually shows modest (about twofold) expansion and the thymus is markedly enlarged in some mice. The enlarged lymphoid organs are replete with lymphoblasts and the blood contains large numbers of similar cells. Variable invasion of other tissues is often observed. The typical pathological diagnosis is therefore multicentric lymphosarcoma (disseminated lymphoma) with associated leukaemia. Occasionally, mice have developed a thymoma without notable involvement of peripheral lymphoid organs and some have succumbed to a bowel obstruction caused by intestinal involution (intussusception) over focal growths of lymphoblasts in the wall. The typical pathology is somewhat like that induced by Abelson murine leukaemia virus³⁴.

Transplantation into syngeneic mice established that the enlarged lymphoid organs of *E_μ-myc* mice represented frank tumours rather than massive proliferations of 'normal' cells. Rapidly growing tumours arose in the (C57×SJL)_{F1} hybrid recipients a few weeks after injection of 10⁵-10⁶ cells from each of 10 *E_μ-myc* mice, including all those in Table 1. Indeed, 10² cells from one tumour tested at lower cell numbers (4Met in Table 1) initiated tumours within 2-3 weeks. The disease in *E_μ-myc* mice is patently highly malignant.

Tumours from single B-lymphoid clones

We wished to identify the cell lineage(s) from which the tumours were derived. The pathology was highly suggestive of a lymphoid disease but did not rule out myeloid involvement. Transcriptional studies of the *μ* constant-region (*C_μ*) locus³⁵⁻³⁷ and gene transfer studies^{7,24} suggest that *E_μ* can operate in many T lymphocytes and certain other haematopoietic cells as well as B cells, but all the tumours so far analysed in detail are B lymphoid in origin. All cell lines derived from them are non-adherent and have an absolute requirement for 2-mercaptoethanol, a strong indication of lymphoid rather than myeloid origin. As expected for B cells, all tumours and cell lines displayed rearrangement and expression of the *IgH* and/or *κ* loci, as illustrated in Figs 3 and 4 and summarized in Table 1 for the tumours of five mice. Arguing against a T-cell origin, no tumour so far studied has displayed the Thy-1 surface antigen, nor exhibited rearrangement or transcription of the T-cell antigen receptor *β* locus (data not shown). Moreover, since the *κ* locus (unlike the *H* locus) appears to be transcriptionally silent in T lymphocytes (refs 6, 38 and our unpublished results), the presence of *κ*-locus transcripts in all the *E_μ-myc* tumour cell lines (see below) strongly favours a B-lymphoid origin.

Table 1 Representative tumours arising in E_{μ} -myc transgenic mice

Pathology	Cell line	Ig genes†		Ig RNA‡		sIg #		Cell type
		H	κ	H	κ	1°	2°	
Mouse 1 (285-5-6)♂								
Multicentric lymphoma/leukaemia with very large pancreatic lymph node	1 Pan*	R1, R2	G	C_{μ} ++	κ^0 +	0	0	Pre-B clone A
	1 Bra*	R3	G	C_{μ} ++	κ^0 +	0	0	Pre-B clone B
Mouse 2 (285-5-12)♀								
Multicentric lymphoma/leukaemia with thymoma	2 Thy†	R1, R2	G	C_{μ} ++	κ^0 +	0	0	Pre-B clone A
	2 Bra*	R3, G§	G	C_{μ} ++	κ^0 +	ND	0	Pre-B clone B
	2 AxL	R3, G§	G	C_{μ} ++	κ^0 +	0	0	Pre-B clone B
	2 Mes*	R3, G§	ND	C_{μ} ++	κ^0 +	ND	<5	Pre-B clone B
Mouse 3 (285-5-9)♀								
Multicentric lymphoma/leukaemia with intussusception of intestine	3 Mes	R1, R2	R1	μ ++	κ ++	5-10	>90	Pre-B → B clone A
	3 Bra	R1, R2	G	μ ++	κ^0 +	5-10	10**	Pre-B → B clone A
Mouse 4 (292-5-5)♂								
Multicentric lymphoma/leukaemia with enlarged thymus	4 Thy†	R1, R2	R1, R2	(μ , C_{μ}) +++	κ +++	80-90	>90	B clone A
	4 Mes†	R3, R4	R3, R4	(μ , C_{μ}) +++	κ +++	80-90	>90	B clone B
	4 Bra†	R3, R4	ND	(μ , C_{μ}) +++	κ +++	80-90	>90	B clone B
	4 Met†	R3, R4	ND	(μ , C_{μ}) +++	κ +++	80-90	>90	B clone B
Mouse 7 (292-1-1-11)♂								
Multicentric lymphoma/leukaemia with thymoma and lymphoblast infiltration of perinodal fat	7 Mes*	R1, R2	R	C_{μ} ++	κ ++	0	0	Pre-B clone A
	7 BM	R1, R2	R	C_{μ} ++	κ ++	ND	ND	Pre-B clone A
	7 ThyD*	R1, R2	R	C_{μ} ++	κ ++	0	ND	Pre-B clone A
	7 ThyV*	R1, R2	R	C_{μ} ++	ND	ND	ND	Pre-B clone A
	7 Spl*	R1, R2	R	C_{μ} ++	ND	ND	ND	Pre-B clone A

Mice specified here were first- or second-generation descendants of three primary transgenic mice (285-5, 292-5 and 292-1) bred with normal (C57BL × SJL)_{F1} hybrids. Sick mice killed by exsanguination under anaesthesia were dissected for assessment of gross pathology. Tissue sections were stained with haematoxylin and eosin and blood smears stained with Giemsa for histopathological assessment. Cell lines are designated by their origin as tumours in the pancreatic (Pan), brachial (Bra), axillary (AxL), mesenteric (Mes) lymph nodes; diffuse mediastinal tissue (Met), thymus (Thy), spleen (Spl) or bone marrow (BM). ThyD and ThyV derived from discrete masses situated dorsally and ventrally in the position of normal thymus. ND, not determined.

* Primary tumour and cell line shown to have equivalent J_H rearrangements.

† Primary tumour oligoclonal but dominated by the single clone present in cell line.

‡ Rearrangement of J_H alleles was determined by Southern blot analysis of *Eco*RI digests using ³²P-labelled probe A (Fig. 3), while J_{κ} analysis used *Bam*HI digests and probe C (Fig. 4, top). G indicates a fragment the same size as that in germline (liver) DNA from C57BL or SJL mice, while R1-R4 indicate rearranged fragments of different sizes.

§ Other rearranged J_H fragments were present in minor yield. We are investigating whether the fragment in germline position is truly unrearranged.

|| Four rearranged bands were detected and their relationship to each other is under investigation.

* μ and κ denote authentic μ and κ mRNA, while C_{μ} denotes all other C_{μ} -bearing RNA species (see refs 35, 37, 39) and κ^0 denotes transcripts of the unrearranged κ locus (see Fig. 4). +++ indicates a level comparable to that in a conventional B lymphoma (WEHI-231), and ++ and + about one-third and one-tenth that level.

* % Cells with surface immunoglobulin (sIg) in either the primary tumour (1°) or the cell line derived from it (2°).

** Growth used for RNA and DNA analysis was 10% sIg⁺, but the line subsequently progressed to >90% sIg⁺.

Although E_{μ} -driven *myc* expression might be expected to trigger proliferation of the entire B-cell compartment, the simplicity of the *IgH* locus rearrangement patterns (Fig. 3) provided strong evidence that most tumours are monoclonal. Most contain two fragments bearing a heavy-chain joining region (J_H) in equimolar yield but lack the 6.5-kb 'germline' fragment found in liver (G), precisely as expected for a clonal lymphoid cell line that has undergone diversity-joining region (DJ) or variable-diversity-joining region (VDJ) recombination at both *IgH* alleles. The apparent single rearranged fragment in tumours such as 1BraC probably reflects two co-migrating fragments. While a few primary tumours (for example, 2AxL, Table 1) contained four to six rearranged J_H fragments, indicative of oligoclonality, one or two bands always strongly predominated.

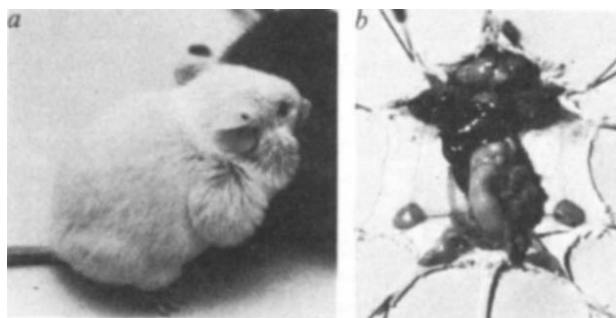


Fig. 2 a, Fourth-generation E_{μ} -myc mouse, 11 weeks old, with swellings due to grossly enlarged lymph nodes. b, Interior of same mouse, displaying gross bilateral enlargement of (from the top) cervical, axillary, brachial, mesenteric and inguinal lymph nodes.

The J_H rearrangement pattern also confirmed that the cultured line (C) represented either the sole or very dominant clone within the primary tumour (T) mass (compare C and T for several tumours in Fig. 3).

The tumour masses at different sites in a *myc* mouse are frequently derived from the same clone. In mouse 7, for example, tumours from five different sites displayed identical J_H rearrangements (Fig. 3, Table 1) and even the liver had been invaded by this clone (Fig. 3). Some mice, however, appeared to bear more than one independent tumour (Table 1). For example, the pancreatic and brachial lymph node tumours in mouse 1 (1Pan and 1Bra) had different J_H rearrangements, while the thymic tumours in mice 2 and 4 differed from the lymph node tumours (Fig. 3). These differences may reflect independent transformation events, or maturation of a single primary clone by further rearrangement, such as recombination of V with DJ segments.

B-lymphocyte transformation

The pre-B cell, the earliest defined stage in B-cell ontogeny, displays rearrangement at the heavy-chain locus (DJ or VDJ) but lacks surface immunoglobulin (sIg), usually because the κ or λ light chain loci are unrearranged. Fusion of a V_{κ} gene with J_{κ} (or, more rarely, V_{λ} with J_{λ}) permits κ or λ expression and progression to the mature (sIg⁺) B cell. Although *c-myc* has been implicated only in tumours of mature B cells and plasma cells, the E_{μ} -myc tumours include some with pre-B characteristics, others that represent mature B cells and one that progressed from the pre-B to the B-cell stage (Table 1). Hence, *myc*-induced tumorigenesis is not stage-specific.

Tumours of pre-B type developed in mice 1, 2 and 7 (Table 1). No sIg was present, and μ_m messenger RNA, which encodes the membrane form of μ -chain, was not detected by blot

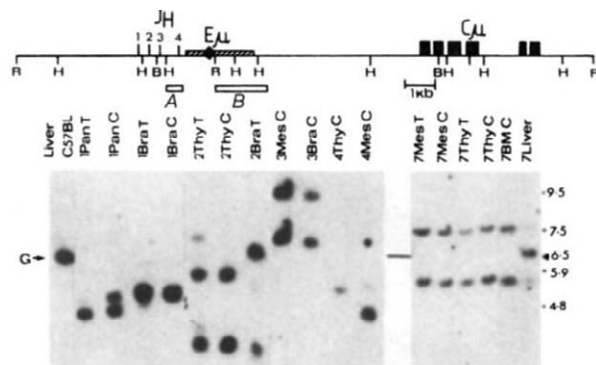


Fig. 3 Rearrangement at the immunoglobulin heavy-chain locus in tumours arising in E_{μ} -myc mice. The diagram indicates the structure of the J_H - C_{μ} locus before rearrangement (germline configuration), with exons indicated as solid boxes, and the enhancer (E_{μ}) as a diamond. Hatched area is the region inserted 5' to c -myc in the E_{μ} -myc construct, and Eco RI (R), Bam HI (B) and $Hind$ III sites are shown. The Southern blot shows J_H rearrangements revealed in Eco RI-cut DNA by hybridization with 32 P-labelled probe A, a 450-bp $Hind$ III/ Nae I fragment, subcloned to eliminate sequences within the E_{μ} -myc insert. An additional fragment detected by probe A in 4Thy (and weakly in 4Mes) is too small (2.8 kb) to be shown by this autoradiograph. An arrow marks the position of the unrearranged J_H -bearing fragment (G) and sizes of marker fragments are given in kilobase pairs (kb). Probe B, a 1.5-kb Eco RI/ Hha I fragment, is the S_{μ} probe used in Fig. 5.

hybridization with a C_{μ} probe (Table 1). As will be documented elsewhere, the C_{μ} -bearing RNAs in these lines are those expected³⁵⁻⁴¹ from either partially rearranged (DJ_H) or unrearranged heavy-chain locus alleles. The tumour in mouse 7 is of an unusual pre-B type, having a κ rearrangement and κ mRNA without μ_m mRNA (Table 1).

Although the cell lines from mice 1 and 2 lack κ rearrangement (Table 1), we found not only the known 8-kb transcript of the germline C_{κ} locus⁴², which initiates upstream of J_{κ} (Fig. 4, top), and a 0.8-kb species⁴³, but also an ~1.1-kb species, the size of κ mRNA (Fig. 4a). Unlike κ mRNA, however, this species bears sequences upstream of J_{κ} , since it hybridizes with probe A + B (Fig. 4b) and probe A alone (not shown). We therefore suggest that processing of the 8-kb RNA generates this previously unrecognized⁴² κ RNA species (Fig. 4, top).

An intriguing tumour that progressed from a pre-B to a B cell developed in mouse 3. The J_H rearrangement patterns assign its mesenteric and brachial lymph node tumours (3Mes and 3Bra) to the same clone (Fig. 3), but only 3Mes had a J_{κ} rearrangement (Table 1). However, after only a few weeks in culture, over 90% of 3Mes cells became sIg⁺, as later occurred for 3Bra. We conclude that this leukaemic pre-B clone generates mature B-cell progeny, as can a few lymphomas induced by Abelson murine leukaemia virus⁴⁴. Thus a myc -induced tumour need not be 'frozen' at one stage of differentiation.

Expression of myc gene

Abundant c -myc mRNA was detected in all the E_{μ} -myc cell lines examined, as illustrated for six lines in Fig. 5a. The levels in all the lines, which included tumours from three separate E_{μ} -myc lineages, were comparable and fell within the upper range of that we have observed in plasmacytomas^{45,46}. Due to the 0.6-kb Φ X174 insert within the myc 3' untranslated region (Fig. 1), all the lines contained an ~3-kb myc mRNA, which also hybridized to the appropriate Φ X174 probe (not shown), whereas a normal, unrearranged myc gene yields ~2.3-kb transcripts, as shown for plasmacytoma ABPC103 and B lymphoma WEHI-231 in Fig. 5a. Significantly, no normal c -myc mRNA was detectable in any E_{μ} -myc cell line, even after lengthy autoradiographic exposures (for example, Fig. 5b). Moreover, treatment of four of the lines with the B-cell mitogen lipopolysaccharide (LPS) at 20 μ g ml⁻¹ for 24 or 48 h did not induce normal

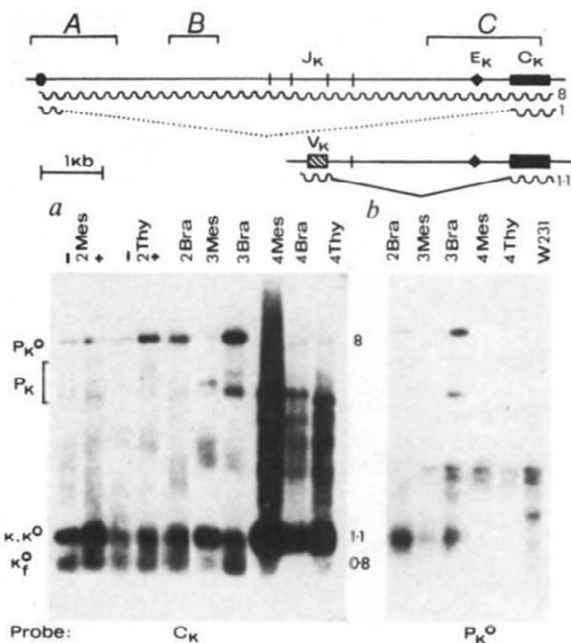


Fig. 4 Transcripts of the κ locus in lymphoma cell lines from E_{μ} -myc mice. The diagram shows that transcripts from the germline C_{κ} locus (top) include a previously described⁴² 8-kb unprocessed (nuclear) RNA initiated at a promoter (filled circle) 3.2 kb upstream from J_{κ} and a proposed spliced ~1.1-kb product ($\kappa^?$) derived from it (see text), while an assembled $V_{\kappa}J_{\kappa}C_{\kappa}$ gene yields 1.1-kb κ mRNA. **a**, C_{κ} -bearing transcripts in the tumour lines. + and - denote cultures grown for 24-48 h in the presence (+) or absence (-) of 20 μ g ml⁻¹ bacterial LPS. The C_{κ} probe was probe C (top), a subcloned 1.5-kb $Hind$ III/ Bgl II fragment. $\kappa^?$ denotes a 0.8-kb C_{κ} -bearing transcript proposed to be derived from a germline allele (see text), while κ^o denotes the unprocessed precursor of authentic κ mRNA. **b**, Transcripts revealed with upstream probes (a mixture of probes A + B above), showing that an ~1.1-kb poly(A)⁺ RNA bears upstream sequences. Probe A alone also labelled the 1.1- and 8-kb species. Probe A is a 1.4-kb $Hind$ III/ Xba I fragment and probe B a 0.7-kb $Hind$ III fragment (see Fig. 1 in ref. 42), prepared from a 12.7-kb Bam HI clone kindly provided by M. Weigert. These probes are free of C_{κ} sequences because they did not reveal a 1.1-kb species in WEHI-231 (W231), which contains abundant κ mRNA, nor in several plasmacytoma lines in which both alleles are rearranged (not shown).

Methods. Tumour lines were established in Dulbecco's modified Eagle's medium supplemented with 50 μ M 2-mercaptoethanol, 100 μ M asparagine and 10% fetal calf serum. Within a few weeks of initiation into culture, the cells were grown in roller bottles and collected while in exponential growth phase ($1-2 \times 10^6$ cells ml⁻¹). Poly(A)⁺ total cellular RNA was isolated from ~10⁹ cells by a method⁵⁴ involving digestion with proteinase K and oligo-dT cellulose chromatography. RNA (2 μ g) heated for 5 min at 65 °C in buffered 2 M formaldehyde/50% formamide was fractionated electrophoretically on a 1.2% agarose/2 M formaldehyde gel and blotted on to nitrocellulose⁵⁵. Hybridization was carried out overnight in 50% formamide/5 × SSC (SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0) at 42 °C, and washing in 2 × SSC at 65 °C.

c -myc transcripts (or alter E_{μ} -myc expression), although shorter times might be needed to reveal the c -myc stimulation observed in normal B cells⁴⁷. At least three of the cell lines were clearly responsive to LPS, since their growth rate increased and the levels of some immunoglobulin RNAs increased slightly (Fig. 4 and our unpublished results). The absence of normal c -myc transcripts favours a feedback control model for myc expression (see below).

Two classes of myc RNA of similar size are apparently made from the E_{μ} -myc insert (Fig. 5, top). One class bears myc exon 1 sequences (Fig. 5c) and appears to initiate at the normal myc promoters P_1 and P_2 , judging by S_1 nuclease protection results (W.A., unpublished results). The second class, apparently of

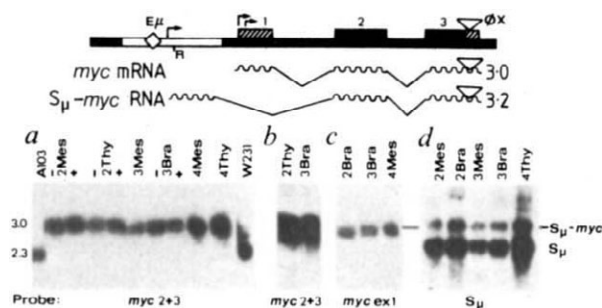


Fig. 5 Transcripts of the *myc* gene in lymphoma cell lines from E_{μ} -*myc* mice. Proposed transcripts from the E_{μ} -*myc* insert are shown at the top (see text). *a*, *myc* transcripts in transgenic lines showing absence of the normal ~2.3-kb *myc* mRNA present in ABPC103 (A103) and WEHI-231, whether the cells were treated with LPS (+) or untreated (-). *b*, Longer exposure of two lanes from *a* (3 days rather than 8 h). *c*, Transcripts bearing exon 1 sequences. *d*, Transcripts bearing S_{μ} sequences, indicating the proposed S_{μ} -(*myc* exons 2+3) hybrid RNA (S_{μ} -*myc*). The *myc* 2+3 probe was a 1.7-kb *Xho*I fragment from a murine *myc* complementary DNA clone kindly provided by K. Marcu; *myc* ex 1, a *Bam*HI/*Xho*I fragment from exon 1 (see map in ref. 45); S_{μ} , a unique-sequence 1.5-kb *Eco*RI/*Hha*I fragment indicated as bar *B* in Fig. 3 (top).

comparable abundance, may have a 5' segment equivalent to that on certain ' S_{μ} ' RNAs, which bear sequences extending ~0.7 kb downstream from E_{μ} , spliced to C_{μ} (ref. 39). A μ switch region (S_{μ})-*myc* hybrid transcript would explain the novel ~3.0-kb RNA species that hybridizes in all the tumour lines to an S_{μ} probe (Fig. 5d) but not to a C_{μ} probe (data not shown). Splicing of the ~0.7-kb S_{μ} sequence to *myc* exon 2 (where the first known splice acceptor occurs), would yield a *myc* RNA marginally larger than the species bearing the normal 0.55-kb *myc* exon 1 (3.2 kb compared with 3.0 kb) and indeed the S_{μ} -bearing species is slightly larger than that detected by a *myc* exon 1 probe on the same filter. The S_{μ} -*myc* RNA is unlikely to encode an altered *myc* polypeptide, because all the ATG sequences within the relevant segment of S_{μ} are followed shortly by stop codons.

Discussion

The novel lymphoid neoplasia described here demonstrates dramatically that subjugation of *c-myc* expression to the immunoglobulin μ or κ enhancer converts this proto-oncogene into a potent leukaemogenic agent *in vivo*. Presumably the malignancy is a consequence of high constitutive *myc* expression within the lymphoid compartment, forced by these enhancers, which are activated by lymphoid factors^{21-23,27,28}. In primary transgenic animals, the E_{μ} -*myc* and E_{κ} -SV-*myc* constructs induced an equivalent pathology, but the E_{μ} -*myc* gene gave a higher tumour incidence and somewhat shorter latent period. This might reflect the greater enhancer activity of E_{μ} over E_{κ} with certain promoters²⁸, or a larger pool of susceptible cells, since E_{μ} is probably activated earlier in lymphoid ontogeny than E_{κ} .

The predisposition to neoplasia in the transgenic lines of E_{μ} -*myc* mice is nearly absolute: over four generations, more than 100 mice involving eight lineages have succumbed within a few months of birth. The typical disease is an aggressive multifocal lymphoma/leukaemia involving lymphoid organs, including the bone marrow, and often invading other tissues. To date, all tumours studied in detail are of the B-lymphoid lineage, but the indications that the E_{μ} enhancer is also active in other haematopoietic cells^{7,35,36,38} leave open the possibility that malignancies of T lymphocytes and myeloid cells arise at a lower frequency. The induction of tumours representing pre-B as well as B cells indicates that *myc*-promoted tumorigenicity is not restricted to one stage.

Our observation that the normal endogenous *myc* genes of the E_{μ} -*myc* tumour lines are silent (Fig. 5) is highly reminiscent

of findings regarding *myc* translocations¹⁸⁻²⁰: in almost all murine plasmacytomas and Burkitt's lymphomas, only the translocated *myc* allele is active^{45,46,48}. One model to account for such results proposes that the normal *myc* gene is subject to negative feedback control by an excess of the *myc* polypeptide or products induced by it^{18,25}. Translocation to the *IgH* locus, or the influence of the E_{μ} enhancer, frees that *c-myc* gene from this restraint, and the ensuing constitutive *myc* expression silences the unaltered allele(s). An alternative model suggests that *c-myc* would not be expressed in a normal cell at the stage of B-cell maturation equivalent to the tumour^{19,49}. A corollary of this model is that *c-myc* expression should be tumorigenic only at those stages where it is normally silent. Judging from Burkitt's lymphomas and murine plasmacytomas, the susceptible stage(s) would be relatively late in B-cell ontogeny, either *slg*⁺ B cells or plasma cells. Our evidence that *c-myc* can participate in the transformation of pre-B cells as well as mature B cells argues against this model but is consistent with feedback control.

It has been possible to argue that the major effect of most *myc* translocations is to dissociate the coding region from a negative regulatory element within *myc* exon 1 or its 5'-flanking region, the immunoglobulin locus being merely a passive bystander caught up because of its propensity for rearrangement (see refs 18-20). We found, however, that a *myc* construct lacking the entire 5' portion but bearing all the cryptic promoters within intron 1 (*myc* ex 2+3 in Fig. 1) was completely ineffectual *in vivo*, as was the intact *myc* gene, in marked contrast to the E_{μ} and E_{κ} constructs. These results bolster the case that the immunoglobulin loci have a positive regulatory input. The puzzle that only a minority of *myc* translocations leave *myc* and E_{μ} on the same chromosome may be partially resolved by evidence that *IgH* genes remain active *in vivo* even when deletions remove E_{μ} (ref. 50). If E_{μ} is needed to initiate but not maintain an active chromatin configuration within the C_H locus, the active C_H locus may suffice for *myc* activation.

A critical issue in oncology is whether a single oncogene can shift a normal cell to the fully malignant state. The potency *in vivo* of the acute retroviruses, such as those bearing *v-myc*, seems to argue that one oncogene suffices, but *in vitro* studies suggest that transformation of normal cells usually requires particular combinations of active oncogenes, such as a *myc* plus a *ras* gene³⁰. The nearly invariant leukaemogenesis of E_{μ} -*myc* mice might be taken as evidence that an active *myc* gene is sufficient for lymphoid neoplasia, but several observations make us think that other events (presumably genetic) influence tumour development. First, the latent period before any overt signs arise is variable and can be as long as 5 months, whereas we would expect E_{μ} -driven *myc* expression to commence concomitantly with B-cell ontogeny, well before birth. Second, there appears to be a true pre-neoplastic state in these mice, since no malignant cells, as assessed by transplantation, are detectable within the lymphoid organs of young transgenic mice free of overt disease (A.W.H., unpublished results). Finally, we are struck by the fact that many of the tumours are monoclonal (Table 1). Since the E_{μ} -*myc* gene should be active within the entire B-lymphoid compartment, clonality implies that one lymphocyte acquired a decided growth advantage, probably by some still undefined genetic alteration. Conceivably the background of the mice (C57BL×SJL)_{F2} might also have some role, since aged SJL mice are subject to a neoplastic disorder of the lympho-reticular system⁵¹.

If dysregulated *c-myc* expression is insufficient for malignancy, why does it elicit high-frequency tumorigenesis? *myc* expression is normally associated with dividing cells and drops precipitously as the cell enters the resting (G_0) state¹⁸⁻²⁰. Thus, constitutive *myc* expression above a certain threshold may promote cell division at the expense of differentiation. The probability that a given cell will proliferate (self-renew) rather than generate differentiated progeny may be set by the level of *c-myc*, or a balance between *myc* and differentiation-promoting elements. This model predicts that excess *myc* expression in a given

lineage will increase the proportion of cells that are less mature and therefore have greater proliferative capacity. Any accidental event that further increases the proliferation/differentiation ratio in a member of this large vulnerable population then creates the malignant clone.

It already seems clear that the diverse set of B-lymphoid cell lines generated by E_{μ} -myc mice will aid studies on lymphoid ontogeny and the mechanisms that regulate immunoglobulin gene rearrangement and expression. Even more significant, the very high predisposition of these mice to neoplasia provides new opportunities for exploring the pre-neoplastic state. It will be intriguing to discover in what ways the haematopoietic popu-

lations have been perturbed by this cellular oncogene freed from its normal restraints.

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Role of acetylcholine receptor subunits in gating of the channel

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The Torpedo and calf acetylcholine receptors and hybrids composed of subunits from the two species have been produced in Xenopus oocytes by the use of the cloned complementary DNAs. Single-channel current measurements indicate that these receptors form channels of similar conductance but with different gating behaviour.

THE nicotinic acetylcholine receptor (AChR) is a transmembrane protein constituting a ligand-gated ionic channel. In the absence of acetylcholine (ACh), the channel is in the closed state. When ACh is bound to the receptor, the channel opens for a few milliseconds, giving rise to an elementary current pulse¹, the size and duration of which can be observed directly by the patch-clamp technique². Biochemical evidence has shown that the AChR from *Torpedo* electroplax consists of four kinds of homologous subunits assembled in a molar stoichiometry of $\alpha_2\beta\gamma\delta$ and that the α -subunit carries the ACh-binding site³⁻⁵. The primary structures of all four subunits of the *Torpedo* electroplax⁶⁻¹¹ and mammalian muscle AChRs¹²⁻¹⁷, the γ - and δ -subunits of the avian AChR¹⁸ and the newly found ϵ -subunit of the calf muscle AChR¹⁹ have been elucidated by cloning and

sequencing complementary DNAs or genomic DNAs encoding these polypeptides. The cloned cDNAs encoding the α -, β -, γ - and δ -subunits of the *Torpedo* AChR can be expressed to produce the functional receptor in *Xenopus* oocytes^{20,21} and analysis of the functional properties of AChR mutants possessing α -subunits altered by site-directed mutagenesis of the cDNA has enabled functional regions of the subunit molecule to be localized²¹.

One possible approach to studying the functional roles of the individual subunits in ion transport and gating of the AChR channel would be to construct hybrid AChR molecules using subunits from different species and to compare their channel properties with those of the parental AChRs. It has been shown recently that the hybrid AChRs composed of the *Torpedo* α -,

Table 1 Functional properties of *Torpedo*, calf and hybrid AChRs

Combination of subunit-specific mRNAs	¹²⁵ I- α -BTX binding (fmol per oocyte)		Whole-cell current				Single-channel current	
	Surface	Extract	AChR channel activity (nA)	Reversal potential (mV)	Voltage sensitivity	Hill coefficient	Conductance (pS)	Average duration (ms)
$\alpha_T\beta_T\gamma_T\delta_T$	7.6 $\pm$ 2.6 (4)	33.7 $\pm$ 19.1 (4)	23 $\pm$ 10 (8)	-4.0 $\pm$ 5.8 (8)	1.0 $\pm$ 0.05 (4)	1.7 $\pm$ 0.2 (4)	42 $\pm$ 2 (5)	0.6 $\pm$ 0.2 (3)
$\alpha_C\beta_C\gamma_C\delta_C$	7.9 $\pm$ 2.6 (4)	84.9 $\pm$ 32.9 (4)	1,600 $\pm$ 1,250 (10)	-11.0 $\pm$ 2.9 (3)	3.1* (2)	1.6† (2)	40 $\pm$ 0.3 (3)	7.6 $\pm$ 1.4 (4)
$\alpha_C\beta_T\gamma_T\delta_T$	10.4 $\pm$ 3.0 (4)	104.2 $\pm$ 36.0 (4)	80 $\pm$ 45 (6)	-6.9 $\pm$ 2.9 (5)	1.0 $\pm$ 0.05 (3)	1.8 (1)	42 $\pm$ 2 (3)	2.5 $\pm$ 0.6 (4)
$\alpha_T\beta_C\gamma_T\delta_T$	0.1 $\pm$ 0.1 (4)	9.1 $\pm$ 5.2 (4)	<1 (12)	—	—	—	—	—
$\alpha_T\beta_T\gamma_C\delta_T$	0.3 $\pm$ 0.2 (4)	9.5 $\pm$ 6.7 (4)	<1 (5)	—	—	—	—	—
$\alpha_T\beta_T\gamma_T\delta_C$	5.4 $\pm$ 3.0 (4)	15.2 $\pm$ 5.1 (4)	1,400 $\pm$ 490 (9)	-1.4 $\pm$ 2.5 (8)	2.0 $\pm$ 0.4 (6)	1.7‡ (2)	35 $\pm$ 3 (3)	8.6 $\pm$ 1.3 (3)

Xenopus laevis oocytes were injected with various combinations of *Torpedo* (indicated by the suffix T) and calf (indicated by the suffix C) AChR subunit-specific mRNAs as specified; molar ratio of the α -, β -, γ - and δ -subunit-specific mRNAs, 2:1:1:1; total mRNA concentration, 100–140 ng μ l⁻¹; average volume injected per oocyte, 20–50 nl. The oocytes were incubated for 2–4 days in conditions described previously²⁰ before being tested for α -BTX binding activity or response to ACh. Data are given as means $\pm$ s.d. Numbers in parentheses refer to the number of experiments for α -BTX binding activity (pools of 10–20 oocytes used in each experiment) or the number of oocytes or membrane patches for current measurements. For assay of the toxin-binding activity on the cell surface³², intact oocytes were incubated at room temperature for 3 h with 2 nM ¹²⁵I- α -BTX (213 Ci mmol⁻¹) in modified Barth's medium³⁴ containing 1 mg ml⁻¹ bovine serum albumin. The toxin-binding activity in cell extracts was determined by the antibody precipitation assay as described previously^{20,21}, using the monoclonal antibody reactive with the calf AChR (mAb210) for oocytes injected with the subunit-specific mRNA combination $\alpha_C\beta_C\gamma_C\delta_C$ or $\alpha_C\beta_T\gamma_T\delta_T$, or rabbit antiserum to the *T. californica* AChR for oocytes injected with the other mRNA combinations; incubation was at room temperature for 3 h in the presence of 2 nM ¹²⁵I- α -BTX. AChR channel activity represents membrane currents in response to bath application of 0.1 μ M ACh. The membrane potential was voltage-clamped at -70 mV. Oocytes injected with the subunit-specific mRNA combination $\alpha_T\beta_C\gamma_T\delta_T$ or $\alpha_T\beta_T\gamma_C\delta_T$ did not respond to 0.1 μ M ACh, but they responded to 0.5 μ M ACh, the average channel activity being 18 $\pm$ 22 nA(7) and 5 $\pm$ 3 nA(3), respectively. Reversal potentials were determined as described in Fig. 1 legend. The voltage sensitivity was quantified as the ratio of the slope conductances at -100 mV and 0 mV membrane potential, measured in Ringer's solution with 1.0 mM or 1.8 mM Mg²⁺ replacing Ca²⁺ (ref. 21). Conductance values represent slope conductances. Mean current amplitudes were measured at least at three different membrane potentials, ranging from -120 mV to -20 mV, in each experiment. The single-channel conductance was obtained as the slope of the *i*-*V* relation determined by linear regression. The average durations of single channel currents are defined as follows. Single-channel current burst durations were measured as any sequence of events separated by less than a critical closed interval of 1 ms. The distributions of such bursts were fitted satisfactorily only by the sum of two exponential components, except for some measurements of the *Torpedo* AChR (see text). The average current durations given represent the slower component. In all cases, the slower component represented more than 70% of the area of the distribution. Averages are from experiments at -110 mV to -70 mV membrane potential.

* Mean of 2.8 and 3.4; † mean of 1.45 and 1.78; ‡ mean of 1.55 and 1.94.

β - and δ -subunits and either the calf γ - or ϵ -subunit are functional when expressed in *Xenopus* oocytes¹⁹. Here we report conductance and gating properties of the *Torpedo* and calf AChRs as well as of the hybrid AChRs in which the α - or δ -subunit of the *Torpedo* AChR is replaced by the corresponding subunit of the calf AChR. Substitution of the calf δ -subunit in the *Torpedo* AChR alters the gating behaviour of the channel drastically, making it similar to the calf AChR channel. Substitution of the calf α -subunit has less effect on the gating properties of the *Torpedo* AChR channel. The conductance of the channel is not significantly affected by either substitution.

Torpedo and calf AChR channels

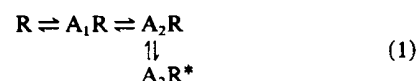
Activity of AChR channels in oocytes. The mRNAs specific for the α -, β -, γ - and δ -subunits of the *Torpedo californica* electroplax and calf muscle AChRs were synthesized *in vitro*^{22,23} using the respective cDNA templates (see refs 19, 21 and Fig. 1 legend). The four subunit-specific mRNAs for the *Torpedo* or calf AChR were combined and introduced into *Xenopus* oocytes by microinjection. The oocytes injected with the calf mRNAs responded to ACh much more strongly than did the oocytes injected with the *Torpedo* mRNAs (Fig. 1a, b), the average membrane currents activated by 0.1 μ M ACh being 1,600 nA and 23 nA, respectively (Table 1). This large difference in membrane current cannot be accounted for by different densities of the calf and *Torpedo* AChRs expressed in the oocyte membrane because the binding activity of α -bungarotoxin (α -BTX) on the cell surface did not differ significantly between the two groups of oocytes (Table 1). These observations imply some functional differences between the calf and *Torpedo* AChR channels.

The current through a membrane (I_m) is given by $I_m = nip(o)$, where n represents the number of channels, i the current through a single channel at a given membrane voltage, and $p(o)$ the probability of the channel being open. Because it can be assumed that the number of channels does not vary significantly between the two groups of oocytes, the difference in channel activity

could reflect a larger elementary current flowing through a single calf AChR channel, or it could reflect a higher open probability of the channel at the given ACh concentration. The following whole-cell and single-channel current measurements were designed to decide between these possibilities.

Figure 1c, d shows current-voltage (*I*-*V*) relations of the ACh-activated current determined in oocytes injected with either the four *Torpedo* or the four calf subunit-specific mRNAs. The reversal potential of the ACh-activated current was -11 to -4 mV in both groups of oocytes (Table 1), suggesting that both the *Torpedo* and the calf AChR channels have similar ion selectivity. A marked difference was observed, however, in the shape of the *I*-*V* relation. In oocytes implanted with the *Torpedo* AChR, the *I*-*V* relation was almost linear, but was curved for oocytes implanted with the calf AChR, indicating an inward rectifying conductance similar to that of frog endplate channels^{24,25}.

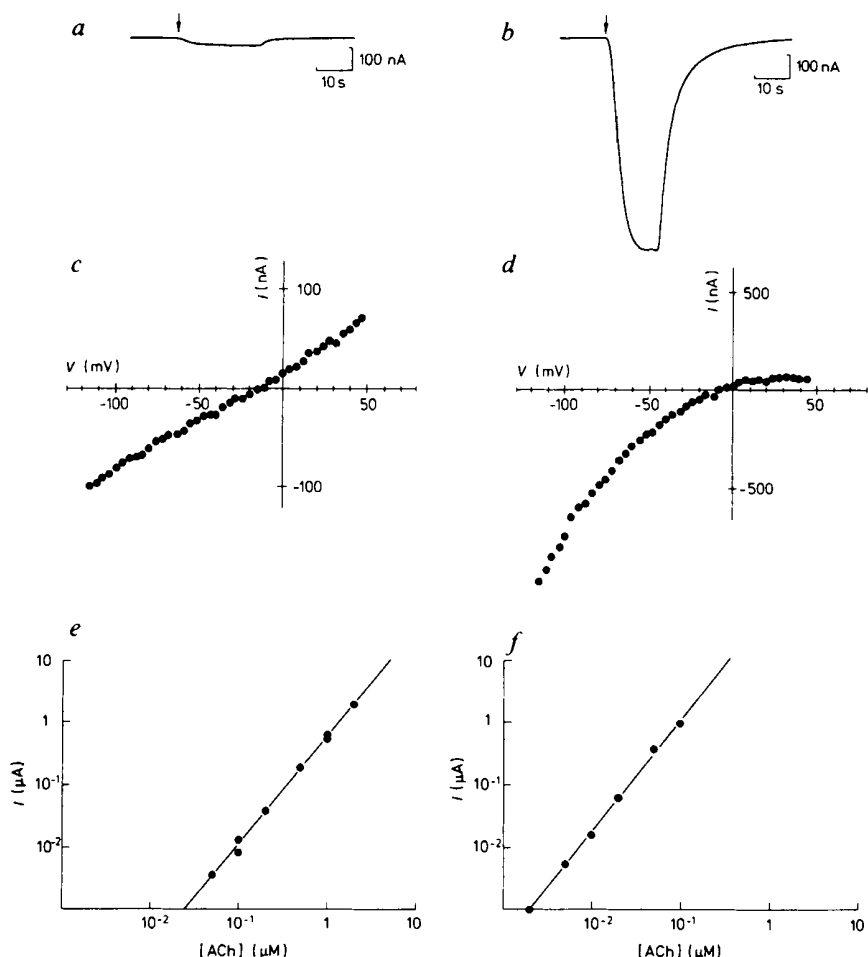
The relation between ACh concentration and membrane current was supralinear, with a Hill coefficient of 1.6–1.7 for both groups of oocytes (Fig. 1e, f, Table 1). This indicates that the *Torpedo* and calf AChRs are comparable in stoichiometry of ACh binding and that the large difference in oocyte channel activity is probably not due to a difference in agonist-receptor stoichiometry. A plausible reaction mechanism for the opening of AChR channels in muscle assumes that channel opening occurs from the bi-liganded receptor²⁶. The agonist-receptor interaction is described by



where R is the resting receptor, A_1R and A_2R are the mono-liganded and bi-liganded receptors, respectively, and the asterisk indicates the open channel state. This simplified reaction scheme does not take other reaction pathways into account. However, it can describe the supralinear relation between ACh concentra-

Fig. 1 ACh-activated whole-cell currents in oocytes injected with the α -, β -, γ - and δ -subunit-specific mRNAs for *Torpedo* AChR (a, c, e) or calf AChR (b, d, f). a, b, Whole-cell current activated by 0.1 μ M ACh, bath-applied at the time indicated by the arrow. Inward current is downwards. Membrane potential, -70 mV. The current amplitude is 30 nA (a) or 960 nA (b). c, d, *I-V* relation of ACh-activated current. Bath application of 0.5 μ M (c) or 0.1 μ M (d) ACh. e, f, Relation between concentration of ACh and amplitude of whole-cell current response. Double logarithmic coordinates. Linear regression yields a slope of 1.7 (e) or 1.8 (f).

Methods. The pSP65 (ref. 23) recombinants carrying the calf AChR α -, β - or δ -subunit cDNA were constructed as follows. The *Sau96I*(-83)/*RsaI*(57) fragment from pcACR α 40 (ref. 12) and the *RsaI*(57)/*PstI*(108) fragment from pcACR α 18 (ref. 12) were ligated. The resulting 190-base-pair (bp) fragment was ligated with the 3.0-kilobase-pair (kb) *PstI*/*HincII* fragment from pSP65. The ligated DNA fragments were treated with *Escherichia coli* DNA polymerase I (Klenow fragment) in the presence of dGTP and dCTP and ligated. A plasmid (pc α SP1) carrying the nucleotide residues -83 to 107 of the calf AChR α -subunit cDNA was identified by colony hybridization with the above *Sau96I*/*RsaI* fragment and by restriction endonuclease analysis. The *PstI*(108)/*PstI*(428) fragment from pcACR α 18 was inserted into the *PstI* site of pc α SP1 in the same orientation as the SP6 promoter. The 366-bp *EcoRI*/*BglII* fragment from the resulting plasmid, the *BglII*(252)/*XbaI*(1,755) fragment from pcACR α 18 and the 3.0-kb *XbaI*/*EcoRI* fragment from pSP65 were ligated to yield pc α SP2. The 1.9-kb fragment from pcACR α 18 extending from the *XbaI*(1,755) site to the *PvuII* site on the vector DNA, and the 3.0-kb *SmaI*/*XbaI* fragment from pSP64 were ligated. The 1.9-kb *XbaI*/*SacI* fragment from the resulting plasmid, the 1.9-kb *EcoRI*/*XbaI* fragment from pc α SP2 and the 3.0-kb *SacI*/*EcoRI* fragment from pSP65 were ligated to yield pSP α . The 308-bp *AvaII* fragment from pcACR β 325¹³ was treated with Klenow fragment in the presence of dGTP, dCTP and dTTP and cleaved with *AccI*. The resulting 268-bp fragment was ligated with the *AccI*(162)/*BamHI*(598) fragment from pcCR β 110¹³ and the 3.0-kb *BamHI*/*SmaI* fragment from pSP65 to yield pc β SP1. The *BamHI*(598)/*BanI*(1,781) fragment and the 704-bp fragment extending from the *BanI*(1,781) site to the *PvuII* site on the vector DNA, derived from clone pcACR β 110, were ligated with the 3.0-kb *HincII*/*BamHI* fragment from pSP65 to yield pc β SP2. The 719-bp *EcoRI*/*BamHI* fragment from pc β SP1 and the 4.9-kb *BamHI*/*EcoRI* fragment from pc β SP2 were ligated to yield pSP β . The ~290-bp *PstI* fragment from pcACR δ 315¹⁵ was inserted into the *PstI* site of pSP65 in the reverse orientation as the SP6 promoter. This plasmid was cleaved with *HindIII*, treated with *Bal31* exonuclease and cleaved with *PstI*. The resulting ~260-bp fragments were ligated with the 3.0-kb *PstI*/*SmaI* fragment from pSP65. A plasmid (pc δ SP1) carrying a *SmaI* site immediately before the initiation codon was identified by DNA sequencing³³. The ~350-bp *PstI* fragment from pcACR δ 315 was inserted into the *PstI* site of pc δ SP1 in the same orientation as the SP6 promoter to yield pc δ SP2. The ~370-bp fragment from pcACR δ 496¹⁵, extending from *XbaI*(1,485) site to the *PvuII* site on the vector DNA, was ligated with the 3.0-kb *HincII*/*XbaI* fragment from pSP65 to yield pc δ SP3. The 3.3-kb *XbaI*/*EcoRI* fragment from pc δ SP3, the 431 bp *EcoRI*/*AccI* fragment from pc δ SP2 and the *AccI*(351)/*XbaI*(1,485) fragment from pcACR δ 496 were ligated to yield pSP δ . The calf AChR α -, β - and δ -subunit-specific mRNAs were synthesized *in vitro*^{22,23}, using pSP α , pSP β and pSP δ (each cleaved with *HindIII*), respectively, and were capped²². The calf AChR γ -subunit-specific mRNA and the *T. californica* AChR α -, β -, γ - and δ -subunit-specific mRNAs were synthesized as described previously^{19,21}. Microinjection and incubation of oocytes were carried out as described for Table 1. All electrophysiological measurements were performed at 19–21 °C in frog Ringer's solution of the following composition (in mM) unless otherwise specified: NaCl, 115; KCl, 2.5; CaCl₂, 1.8; HEPES (pH 7.2), 10. Atropine (0.5 μ M) was added to all bath solutions to block muscarinic AChRs. Follicular cells were removed from oocytes by treatment with 1 mg ml⁻¹ collagenase (Sigma type 1) in modified Barth's medium³⁴ at room temperature for 1 h. Oocytes were then held in an experimental chamber of 0.5 ml volume through which Ringer's solution was perfused continuously at 5 ml s⁻¹. Oocytes were voltage-clamped with a conventional two-micropipette voltage clamp. The voltage-recording pipette was filled with 3 M KCl, and the current injection pipette with a solution containing 0.25 M CsF, 0.25 M CsCl and 50 mM EGTA. ACh-containing solution was perfused for 10 s through the chamber. *I-V* relations were measured by changing the membrane potential in a rampwise fashion (40 mV s⁻¹) from -120 mV to +50 mV in the absence and presence of low (<1 μ M) ACh concentrations. The difference current was obtained by subtracting the two current records at 4-mV intervals. The reversal potential was obtained by linear interpolation between inward and outward current values measured close (<12 mV) to the reversal potential. All *I-V* relations were determined in extracellular solution where 1.0 mM or 1.8 mM Mg²⁺ replaced Ca²⁺ (ref. 21). The relation between ACh concentration and current amplitude was measured at -70 mV in normal Ringer's solution. ACh (2 nM–2 μ M) was bath-applied for 10 s.



tion and membrane current observed for AChR-implanted oocytes.

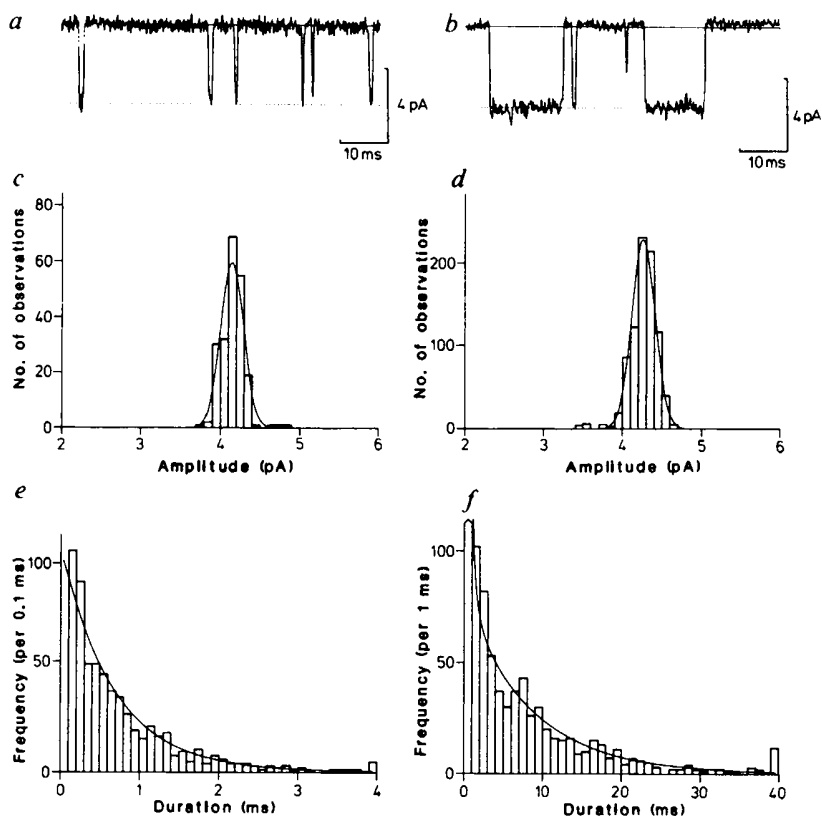
Elementary currents. Figure 2a, b compares ACh-activated single-channel currents recorded from outside-out patches isolated from oocytes injected with either the four *Torpedo* or the four calf subunit-specific mRNAs. In the two patches, the amplitudes of single-channel currents at -100 mV membrane potential were of similar size, that is, 4.1 pA and 4.2 pA for the *Torpedo* and calf AChR channels, respectively (Fig. 2c, d). The single-channel *i-V* relations were linear, both channels exhibiting a slope conductance close to 40 pS (Table 1).

To characterize the ion selectivity of the two classes of AChR channels, we determined the conductance sequence for

monovalent metal cations. In nominally Ca²⁺-free extracellular solution (10 mM EGTA added), the conductance sequence of the *Torpedo* and calf AChR channels was K⁺ > Cs⁺ > Na⁺ > Li⁺. The single-channel slope conductances for these cations, determined at an extracellular cation concentration of 100 mM and a driving potential of -100 mV, were (mean of two patches): 107 pS, 90 pS, 60 pS and 30 pS for the *Torpedo* AChR and 70 pS, 60 pS, 50 pS and 21 pS for the calf AChR. These values are close to the limiting conductances because the conductance did not increase further when the extracellular cation concentration was raised to 200 mM or 300 mM. The half-saturation concentrations were in the range 20–30 mM. The conductance sequence corresponds to the Eisenman sequence III or IV (ref. 27), which

Fig. 2 Properties of ACh-activated single-channel currents recorded from outside-out patches isolated from oocytes implanted with *Torpedo* AChR (a, c, e) or calf AChR (b, d, f). a, b, Records of single-channel currents. Inward current is downwards. Membrane potential, -100 mV. Low-pass filtering at 3 kHz (a) or 2 kHz (b) (-3 dB). At these filter settings the short interruptions of elementary currents occurring in the time range of tens of microseconds^{31,32} are largely attenuated in the records shown. c, d, Distribution of current amplitudes. Membrane potential, -100 mV; mean amplitude, 4.1 ± 0.14 pA (c) or 4.2 ± 0.15 pA (d) (mean $\pm$ s.d.). e, f, Distribution of current durations. Membrane potential, -100 mV. The distribution in e is fitted by a single exponential; decay time constant, 0.61 ms. The distribution in f is fitted by the sum of two exponentials; decay time constants, 0.4 and 8.6 ms. Only the slower component is shown fully in f. The last bin includes durations longer than 4 ms (e) or 40 ms (f). The first bin in f has a roof to indicate that it extends beyond the scale.

Methods. Single-channel currents were measured in outside-out and cell-attached membrane patches³⁵. All experiments were done at 19–21 °C on oocytes bathed in normal frog Ringer's solution. The vitelline membrane covering the oocyte was removed mechanically to allow close contact between pipette and oocyte plasma membrane with a seal resistance >10 G Ω . Mechanical removal of the vitelline membrane was facilitated by placing the oocyte for 5 min in hypertonic 'stripping solution' (in mM: K-aspartate 200; KCl 20; MgCl₂ 1; EGTA 10; HEPES (pH 7.2) 10). The pipette solution in the outside-out patch recording configuration had the following composition (in mM): KCl 20; KF 80; MgCl₂ 1; EGTA 10; HEPES (pH 7.2) 10. Following formation of an outside-out patch, ACh was applied from another pipette the opening of which (≈ 500 μ m diameter) was located close to the membrane patch (~ 100 μ m distance). The ACh concentration in this pipette was 0.1–5 μ M. The conductance sequences for different alkali metal cations were measured in cell-attached patches. Thick-walled patch pipettes of small (≈ 0.5 μ m diameter) tip openings were used. With these pipettes the occurrence of endogenous stretch-activated single-channel currents is significantly reduced (C.M. *et al.*, in preparation). K⁺, Cs⁺, Na⁺ or Li⁺ were present at 100 mM in the pipette solution which contained also 10 mM EGTA and 10 mM HEPES (pH 7.2). Conductance values represent the slope conductance measured at a driving potential of -100 mV. Patch-clamp currents were measured with a LIST EPC-7 amplifier and recorded on magnetic tape (Racal Store 4). After appropriate filtering, current records were digitized at 20–100 μ s per point and analysed on a PDP 11/73 computer. For analysis of the amplitude and duration of single-channel current records of the *Torpedo* AChR, the method of time course fitting³⁶ was used. Currents mediated by the calf and hybrid AChRs were analysed also by the threshold-crossing method³⁶; the threshold was half the full amplitude. Amplitude distributions contained only fully resolved current pulses and were fitted by single Gaussians. Single channel current *i*-*V* relations in outside-out patches were measured in the voltage range -120 to $+50$ mV. Single-channel conductances were determined as slope conductances in the voltage range -120 mV to -20 mV. Distributions of durations were fitted with either one or two exponential components using the maximum likelihood method³⁶. The Patternsearch or the Simplex method was used to obtain the parameters of the distribution³⁶. The average duration of elementary currents in the text and in Table 1 refers to the decay time constant of the slower component.



suggests, in terms of Eisenman's selectivity theory, a relatively weak interaction between the ion in the channel and an anionic binding site on the channel wall in both *Torpedo* and calf AChR.

Whereas the ion selectivity and transport properties of the *Torpedo* and calf AChR channels were comparable, their gating behaviour differed markedly. The average duration of elementary currents flowing through calf AChR channels was much longer than that observed for the *Torpedo* AChR channel. Figure 2e, f shows histograms of current durations at -100 mV membrane potential. Distributions of current durations were fitted satisfactorily only by the sum of two exponential components, except for some measurements of the *Torpedo* AChR channel, where the number of resolved short durations was too small to allow a reliable estimate of the decay time constant of the faster component. The slower component predominated in all experiments and we therefore restricted the analysis to this component. The average duration of single-channel currents was 0.6 ms for *Torpedo* AChR at membrane potentials of -110 mV to -70 mV, in striking contrast to the 7.6 ms obtained for calf AChR (Table 1).

Another difference between the two AChR channels was revealed when the average duration of elementary currents was measured at different membrane potentials. The average current duration of the *Torpedo* AChR channel was not significantly

voltage-dependent; in one experiment, measured at membrane potentials of -50 mV and $+50$ mV in the same membrane patch, they were 0.48 and 0.46 ms, respectively. Elementary currents of the calf AChR channel (Fig. 2f) had average durations of 8.6 ms, 6.8 ms and 3.9 ms at membrane potentials of -100 mV, -50 mV and $+50$ mV, respectively, suggesting that the gating reaction of the calf AChR is voltage-dependent. This probably underlies the inward rectification in the whole-cell *I*-*V* relation for the calf AChR (see Fig. 1d).

Hybrid AChR channels

To determine which subunit is responsible for the marked difference in gating behaviour between the *Torpedo* and calf AChRs, we constructed hybrid AChRs by injecting oocytes with the corresponding combinations of *Torpedo* and calf subunit-specific mRNAs, then analysed the functional properties of the hybrid AChRs.

α -Hybrid AChR. The hybrid AChR formed in oocytes injected with the calf α -subunit-specific mRNA and the *Torpedo* β -, γ - and δ -subunit-specific mRNAs (α -hybrid AChR) showed an average channel activity somewhat higher than that of the *Torpedo* AChR but much lower than that of the calf AChR (Fig. 3a, Table 1). The whole-cell *I*-*V* relation was almost linear, and the reversal potential close to 0 mV (Fig. 3b, Table 1); the dose-

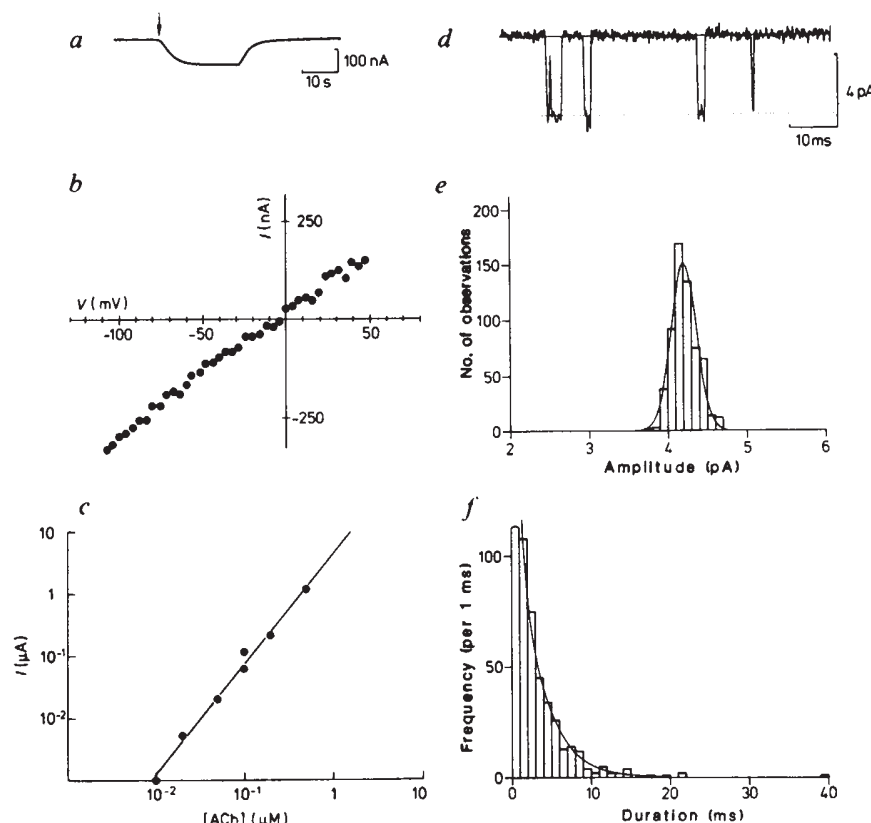


Fig. 3 Whole-cell (*a-c*) and single-channel (*d-f*) current measurements for oocytes implanted with the α -hybrid AChR. *a*, Whole-cell current activated by $0.1 \mu\text{M}$ ACh bath-applied at the time indicated by the arrow. Inward current is downwards. Membrane potential, -70 mV . The current amplitude is 120 nA . Note that the scales are the same as in Fig. 1*a*. *b*, *I-V* relation of ACh-activated current between -120 mV and $+50 \text{ mV}$ membrane potential. *c*, Relation between ACh concentration and whole-cell current response. Double logarithmic coordinates. The slope of the straight line fit is 1.8 . *d*, Single-channel currents. Inward current is downwards. Membrane potential, -100 mV . Low-pass filtering at 2 kHz (-3 dB). *e*, Distribution of current amplitudes. Membrane potential, -100 mV . The mean amplitude is $4.2 \pm 0.16 \text{ pA}$. *f*, Distribution of current durations. Membrane potential, -100 mV . Line represents fit by the sum of two components; decay time constants, 0.41 ms and 3.2 ms . Only the slower component is shown fully. The last bin includes durations $>40 \text{ ms}$.

response curve yielded a Hill coefficient of 1.8 (Fig. 3*c*). The properties of whole-cell currents (Table 1) indicate that the α -hybrid AChR behaves much like the *Torpedo* AChR.

Figure 3*d* shows single-channel current recordings of the α -hybrid AChR, and Fig. 3*e, f* the distribution of current amplitudes and of current durations at -100 mV membrane potential. The channel conductance of this hybrid AChR (42 pS) was virtually the same as that of the parental AChRs (Table 1). The mean duration of single-channel currents of this hybrid (2.5 ms) was longer than that of the *Torpedo* AChR (Table 1). The average duration of single-channel currents of the α -hybrid AChR was essentially independent of membrane potential; the values obtained at membrane potentials of -100 mV and $+50 \text{ mV}$ were 3.2 and 2.7 ms , respectively (Fig. 3*f*).

δ -Hybrid AChR. The hybrid AChR formed in oocytes injected with the calf δ -subunit-specific mRNA and the *Torpedo* α -, β - and γ -subunit-specific mRNAs (δ -hybrid AChR) showed an average channel activity ~ 60 -fold higher than that of the *Torpedo* AChR and comparable to that of the calf AChR (Fig. 4*a*, Table 1). The whole-cell *I-V* relation was curved, indicating that the ACh-activated conductance mediated by the δ -hybrid AChR channel rectifies and the reversal potential was close to 0 mV (Fig. 4*b*, Table 1). The dose-response curve gave a Hill coefficient of 1.7 (Fig. 4*c*, Table 1).

Single-channel current records (Fig. 4*d*) revealed that the channel conductance and gating behaviour of the δ -hybrid AChR are similar to those of the calf AChR. Figure 4*e* and *f* show the distribution of current amplitudes and of current durations at -110 mV membrane potential, respectively. The channel conductance of the δ -hybrid AChR (35 pS) was nearly the same as that of the parental AChRs (Table 1). However, the average duration of elementary currents was 8.6 ms , more than 10-fold longer than that of the *Torpedo* AChR and comparable to that of the calf AChR (Table 1). The average current duration of the δ -hybrid AChR channel was voltage-dependent being 10.3 ms and 3.5 ms at membrane potentials of -110 mV and $+50 \text{ mV}$, respectively (Fig. 4*f*).

β - And γ -hybrid AChRs. Two additional hybrid AChRs were produced by injecting oocytes either with the calf β -subunit-specific mRNA and the *Torpedo* α -, γ - and δ -subunit-specific mRNAs (β -hybrid AChR) or with the calf γ -subunit-specific mRNA and the *Torpedo* α -, β - and δ -subunit-specific mRNAs (γ -hybrid AChR). Oocytes containing these hybrid AChRs showed a very low channel activity (Table 1). This is probably due to an extremely low density of the β - and γ -hybrid AChRs expressed in the oocyte membrane because the α -BTX binding activity on the cell surface was markedly diminished despite the considerable activity found in the cell extract (Table 1). It thus appears that in these hybrids subunit assembly is impaired, forming aberrant complexes. Therefore, we did not investigate channel properties of the β - and γ -hybrid AChRs.

Conclusions

Both *Torpedo* and calf AChR channels, produced in the *Xenopus* oocyte membrane by translation of the α -, β -, γ - and δ -subunit-specific mRNAs, exhibit virtually the same conductance. In Ringer's solution, with Na^+ as the main charge carrier, the conductance is $\sim 40 \text{ pS}$. Any subunit of the *Torpedo* AChR can be replaced by the corresponding subunit of the calf AChR to form functional channels. The conductances of α - and δ -hybrid AChR channels are similar to those of the parental AChR channels. These observations are consistent with the high degree of amino-acid sequence homology between corresponding subunits of the *Torpedo* and calf AChRs⁶⁻¹⁵. Each subunit contains five putative transmembrane segments^{6-9,11-15,21,28,29} which are involved directly in the formation of the channel²¹.

In contrast to the uniform conductance of the *Torpedo*, calf and hybrid AChR channels, their gating behaviour differs widely. The average duration of elementary currents of the calf AChR channel is more than 10-fold that of the *Torpedo* AChR channel. The mean current duration of the α -hybrid AChR channel lies between those of the *Torpedo* and calf AChR channels, while that of currents through the δ -hybrid AChR channel is comparable to that of the calf AChR channel. There-

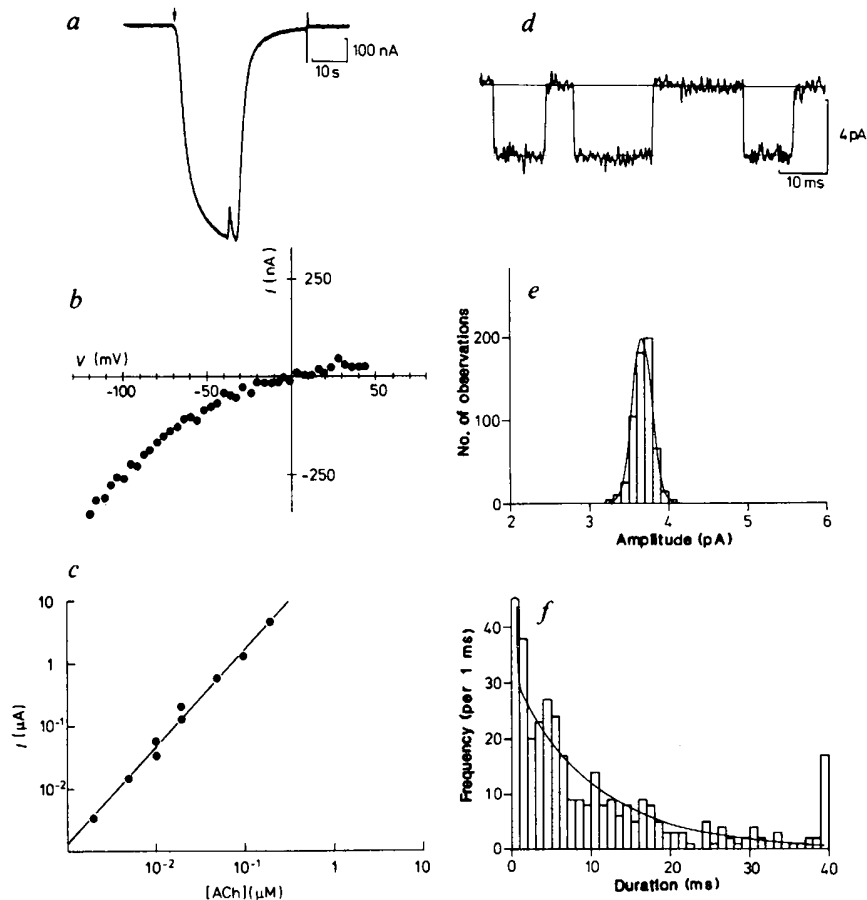


Fig. 4 Whole-cell (*a-c*) and single-channel (*d-f*) current measurements for oocytes implanted with the δ -hybrid AChR. The experimental conditions were the same as for Fig. 3, unless otherwise stated. *a*, ACh-activated whole-cell current. Current amplitude, 940 nA. *b*, I - V relation of ACh-activated current. *c*, Relation between ACh concentration and whole-cell current response. The slope of the straight line fit is 1.6. *d*, Record of single-channel currents. Membrane potential, -110 mV. *e*, Distribution of current amplitudes. Membrane potential, -110 mV. The mean amplitude is 3.7 ± 0.12 pA. *f*, Distribution of current durations. Membrane potential, -110 mV. Decay time constants, 0.14 ms and 10.3 ms.

fore, it seems plausible that presently unidentified functional domains of the calf δ - and α -subunits determine the longer duration of elementary currents.

The average duration of elementary currents through AChR channels is determined, in the framework of the simple two-step reaction mechanism (1) described above, by at least three reaction steps: channel opening, channel closing and dissociation of the agonist. The increased duration of elementary currents in the δ -hybrid and α -hybrid AChR channels, as compared with that of the *Torpedo* AChR channel, could reflect a difference in any of the rate constants governing these steps. The voltage dependence of whole-cell I - V relations and single-channel current durations might give a clue as to which reaction step is different. In frog endplate, the curvature of the whole-cell I - V relation and the voltage sensitivity of the average single-channel current duration are determined by the voltage dependence of

channel closing^{30,31}. The observation that both the calf AChR and the δ -hybrid AChR behave like the frog AChR would then be consistent with the hypothesis that the δ -subunit determines the channel-closing step. The α -hybrid AChR is also characterized by an average current duration somewhat longer than that of the *Torpedo* AChR but which is voltage-insensitive, suggesting that the α -subunit may mainly determine another reaction step, such as channel opening or agonist dissociation.

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Shepherding model for Neptune's arc ring

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An incomplete arc ring has recently been discovered around Neptune¹. Here, a model to explain the confinement of this ring is developed. The ring may be azimuthally confined near a triangular (Trojan) point of an undiscovered satellite of Neptune. Radial diffusion of the ring particles can be prevented by shepherding torques of another moon. Two satellites with diameters of 100–200 km would be sufficient to confine the ring; such moons would be too small to have been photographed from Earth.

The arc ring of Neptune¹ differs substantially from other known planetary rings. Whereas the rings of Jupiter, Saturn and Uranus completely encircle these planets, Neptune's ring appears to be an incomplete arc, spanning only a small fraction of an ellipse. The exact dimensions of the ring are poorly constrained, as it has only been observed by stellar occultation. Detections of the ring at three separate telescopes imply that it is located at a distance of $r \sim 67,000$ km from Neptune's centre, has a width $w \sim 15$ km, an optical depth $\tau \sim 0.08$, and a length of at least 100 km, all assuming that the ring lies in Neptune's equatorial plane¹. Approximately 10 other stellar occultations of the Neptune system have failed to show such a ring, although an event observed at a similar distance from Neptune by two closely spaced telescopes during one occultation, which has been tentatively attributed to a satellite (provisionally named 1981N1)², could possibly be an optically very thick ring. The current detection rate leads to a crude estimate that the arc ring spans $\sim 10\%$ of the circumference around Neptune.

Theories developed to explain the rings of other planets do not fully explain arc rings. If the particles in an arc ring were unconstrained, they would spread out azimuthally because of the shorter orbital periods of particles nearer the planet. An arc ring 15-km wide located 67,000 km from Neptune's centre would spread out to encircle the entire planet in less than 3 yr. Interparticle collisions and, for small particles, Poynting–Robertson drag, would cause radial spreading on a timescale of less than 10^8 yr (ref. 3), which is short compared with the age of the Solar System. Thus, some active confining mechanism is required, unless the arc ring is extremely young.

Torques caused by observed moons are responsible for several sharp boundaries in Saturn's rings^{4–8}. Undetected moonlets are believed to be responsible for confining the uranian rings³, as well as several features in Saturn's rings⁹. Although no discontinuous arc ring is seen (except possibly within the Encke gap in Saturn's A ring⁹), several of the resonantly produced features are eccentric^{4,7,8}. Satellites are thus capable of producing azimuthally variable ring structure.

Jupiter confines the Trojan asteroids to a limited azimuthal range around the stationary triangular (Lagrange) points of the Jupiter–Sun system, L_4 and L_5 . These asteroids are in a 1:1 orbital commensurability with Jupiter, orbiting 60° ahead of and behind the planet. Similar Lagrange point librators share orbits with some of Saturn's many moons^{4,10,11}. One early model of the uranian rings proposed that they were confined in a similar, although less tightly bound, horseshoe resonance lock¹². However, although individual satellites and groups of non-interacting satellites orbit stably in such resonance locks, collisional rings cannot be confined in such a manner. The problem arises because the triangular points are potential maxima (which are only stable because of Coriolis forces¹³), and interparticle

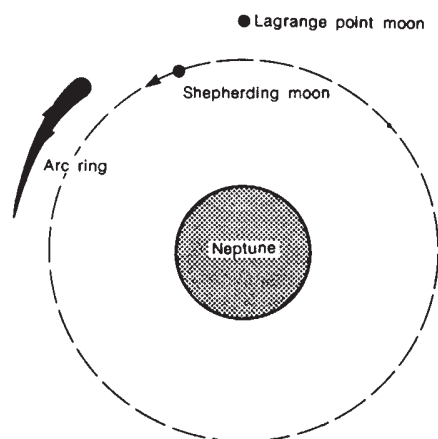


Fig. 1 Neptune's arc ring viewed from above Neptune's pole, as predicted from the present model. The ring appears stationary in the frame rotating with the Lagrange point moon, although the individual ring particles librate slowly about the triangular point in the direct indicated. The frame rotates in the anticlockwise direction with respect to inertial space.

collisions, which dissipate energy, drive particles away from energy maxima. Although a satellite can azimuthally confine ring particles which have semimajor axes (nearly) the same as its own, interparticle collisions and other dissipative effects will cause radial spreading similar to that of an unconfined ringlet.

Saturn's F ring is confined to a radially narrow strand caused by torques from 'shepherding' moons on both sides⁴. Undetected shepherding moons are believed to confine the nine narrow rings of Uranus³. Shepherding is possible because a moon exerts a torque which repels nearby ring material. This torque falls off rapidly with distance from the moon. Ring particles initially orbiting between two moons are thus confined to a ring whose radial width is determined by a balance between diffusive spreading and satellite torques³.

We suggest that the arc ring of Neptune is azimuthally confined near a triangular Lagrange point of an undetected satellite (or, possibly, 1981N1), and radially confined by at least one shepherding moon on a nearby orbit (Fig. 1). One shepherding moon is sufficient because the ring particles' orbits are alternately interior to, and exterior from, that of the confining Lagrange point satellite. The shepherding moon, which may orbit either interior to, or exterior from, the ring, repels all the ring particles. However, when a particle is on the half of its libration cycle which brings it nearer the shepherd, the magnitude of the repulsive torque, which at the time is pushing the ring particle towards the orbit of the Lagrange point moon, is greater than the magnitude of the repulsive torque pushing the particle away from the Lagrange point moon during the other half of its libration cycle. Thus, the shepherd's net effect is to stabilize the 1:1 resonance between the ring particles and the Lagrange point moon. In celestial mechanics terminology, the torque caused by the shepherding satellite acts, on average, to decrease the Jacobi constants of the ring particles, driving them closer to L_4 or L_5 . Note that energy conservation is not violated; the energy lost in inelastic collisions between ring particles is resupplied to the ring from orbital energy of the rings and moons. The shepherding moon is repelled from the ring in the process, but the timescale for this to occur may be longer than the age of the Solar System. (Alternatively, tidal torques on the moons by Neptune may counteract this repulsion.)

The combination of a Lagrange point moon and a shepherding moon on a nearby orbit is thus qualitatively capable of producing the arc ring structure observed around Neptune. Quantitative estimates will now be made which show that Neptune's ring may be confined by moons of sufficiently small size that they would not have been photographically detected from Earth.

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To zero order, the orbits of the ring particles are keplerian ellipses about Neptune. The semimajor axes of these ellipses are perturbed by the lagrangian moon, causing the ring particles to librate about one of the moon's triangular points with periods of the order of 1 yr. Perturbations by the shepherding moon induce eccentricities in the ring particles. The damping of these eccentricities results in a radial drift on a much longer timescale, comparable with the timescale for diffusive spreading of the ring.

The relationship between the radial, δr , and angular, θ , separations of a given ring particle from the Lagrange point satellite is:

$$\frac{3}{4} \left(\frac{\delta r}{r} \right)^2 + \frac{m}{M} \left(\frac{1}{\sin(\theta/2)} - 2 \cos \theta + 2 \right) = C - 3 \quad (1)$$

where r is the satellite's semimajor axis, m is the mass of the Lagrange point moon, M is Neptune's mass, and C is the Jacobi constant¹⁴. The Jacobi constant depends on the amplitude of the particle's libration about the triangular point, and differs for different ring particles. For a given ring particle, the Jacobi 'constant' varies slowly, in response to torques from the shepherding moon and interparticle collisions. The width of a particle's libration at a given angle can be computed from equation (1) to be:

$$w = 2\delta r = \frac{4r}{\sqrt{3}} \left\{ C - 3 - \frac{m}{M} \left(\frac{1}{\sin(\theta/2)} - 2 \cos \theta + 2 \right) \right\}^{1/2} \quad (2)$$

and the length of the arc, l , is:

$$l = r(\theta_2 - \theta_1) \quad (3)$$

where the θ_i are the angles at which $\delta r = 0$ (see equation (1)). Presumably, the shepherding moon confines the ring particles to a maximum value of C (and thus of δr). If the arc ring subtends no more than 10% of the circumference of the satellites orbit, and its maximum width is at least 15 km, then a lower bound on the lagrangian satellite's mass is 4.5×10^{21} g; this bound may be reduced to 0.48×10^{21} g if an arc of 156° (the maximum possible libration amplitude for tadpole orbits) is allowed. Assuming a density of 1 g cm^{-3} , a moon of mass 4.5×10^{21} g would have a diameter ~ 200 km. The tentatively detected moon 1981N1, which presumably has a diameter between 100 and 1,000 km (ref. 2), is thus a candidate for the Lagrange point satellite.

The size(s) of the shepherding satellite(s) are less well constrained. The torque per unit ring mass, T_s , on a ring at radius r caused by a shepherding satellite of mass m_s at radius r_s is^{3,15}:

$$T_s = 0.4 \text{ sign}(r - r_s) \left\{ \frac{Gm_s}{\Omega(r - r_s)^2} \right\}^2 \quad (4)$$

where Ω is the angular frequency of the ring particles. The differential shepherding torque across the ring must balance the viscous and other diffusive torques within the ring (including the extra diffusive torque caused by stirring by the shepherding moon¹⁶). Assuming just one shepherding moon, its mass would be roughly:

$$m_s \sim 3 \times 10^{21} \left(\frac{\nu}{10} \right)^{1/2} \left(\frac{r - r_s}{10^8} \right)^{5/2} \quad (5)$$

where ν is the effective kinematic viscosity of the ring and all quantities are in c.g.s. units. Estimates of ν for Saturn's rings range between 5 and $260 \text{ cm}^2 \text{ s}^{-1}$ (refs 8, 17). Thus, the shepherding moon would be ~ 120 km in diameter if its orbit was 1,000 km from that of the ring; the required diameter would scale slightly slower than linearly with its distance from the ring. An arc ring which has a maximum width $\gg 15$ km would permit a smaller shepherd for a given separation and viscosity; however, it would require a significantly larger Lagrange point satellite. If there were shepherds on both sides of the ring, required masses would decrease by a factor of $\sqrt{2}$.

Thus, Neptune's arc ring may be confined by two nearby moons each ~ 100 – 200 km in diameter. One of the moons would

restrain the ring azimuthally near one of its stable triangular points with Neptune. The other moon, circulating on a different orbit, would confine the ring radially in the standard shepherding manner. Other configurations are also possible. For example, the shepherding moon may be coorbital with the Lagrange point moon, but have a larger libration amplitude than that of the ring. The problem with this latter scenario is that the torque from the ring would drive the shepherd away from the coorbital resonance; either the moons would have to be much more massive than the ring (so the timescale would be very long) or the system would have to be supplied with energy from an external source (such as tidal torque from the planet).

Further observations of Neptune's arc ring by stellar occultations, the Hubble Space Telescope and, in 1989, by the Voyager 2 spacecraft, should greatly increase our knowledge of this newly discovered structure, and determine whether the model presented here is accurate. A search for arc rings orbiting the other giant planets may also prove fruitful.

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Timescale-invariant profile of the type II bursts from the Rapid Burster

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The Rapid Burster is a unique X-ray source which produces rapidly repetitive X-ray bursts. Each rapid burst shows a characteristic structure in its decay part comprising successive peaks. We have now discovered that the structure of each individual burst shows a nearly identical profile except for the timescale, which differs largely from burst to burst; the profile of the decay is timescale invariant. This invariance holds over a wide range of timescales to at least a factor of 30. Furthermore, we have found that the heights of successive peaks as well as the intervals between them decrease with time according to a common arithmetic rule.

The rapidly repetitive bursts from the Rapid Burster are probably due to chopped accretion flow caused by some instabilities¹. Hoffman *et al.*² designated these bursts type II bursts and those interpreted as thermonuclear flashes as type I bursts. Here, we shall discuss the remarkable characteristics of the structure in the decay part of type II bursts from the Rapid Burster.

We observed the rapid burst activities of this source from Tenma in August 1983 (ref. 3) and July 1984. Each burst exhibits

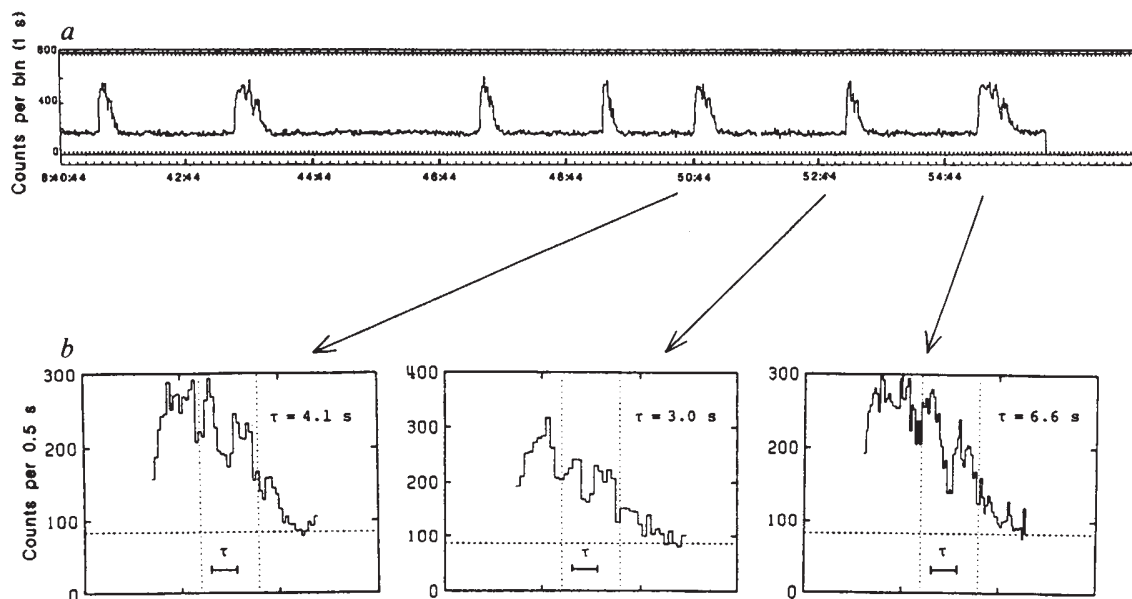


Fig. 1 *a*, A portion of the rapid burst activity (1983). *b*, Detail of the profiles of three bursts with different length. Timescale in these diagrams is in units of the characteristic time τ which is determined from a cross-correlation analysis performed between the two dashed lines for each burst.

a significant structure in the decay part. Long flat-topped bursts with durations exceeding 40 s show one or two humps in the decay. Bursts of shorter duration commonly comprise more than two peaks (Fig. 1). Clearly, the timescale of the structure depends on the burst duration; the longer the burst duration, the slower is the excursion of the decay structure.

There is a striking similarity in the form of the decay structure between bursts, except that the timescale of the structure differs from burst to burst. In order to examine the similarity in more detail, we performed a cross-correlation analysis of the decay profile between bursts by varying timescales, and determined the relative timescale for each burst. Cross-correlation was taken between the dashed lines shown in Fig. 1. For convenience, we define the characteristic time τ for each burst as being the interval between the first and second peaks in the decay, as also indicated in Fig. 1.

We then constructed composite profiles from many bursts by normalizing the characteristic times, as shown in Fig. 2 for different ranges of τ . The number of bursts superimposed is indicated on each figure.

In Figs 1 and 2 we see that the decay structure is essentially independent of the timescale. In other words, the profile of the decay is timescale invariant and can be expressed by a single function $F(t/\tau)$ with time t . Obviously, the profile is not of a simple damping oscillation but more complex. For instance, the second and the third peaks are close together and form a broad hump. Such fine detail is not eliminated by superimposing many bursts. This fact implies that, independently of the length of timescale of each burst, the structure is intrinsic and solid, as in the case of the X-ray pulsar profiles. Furthermore, we stress that the timescale invariance of the decay profile holds for a wide range of the characteristic time τ from 9 to 0.3 s, the shortest limit with our time resolution.

As regards the detail of the decay structure, we note that there is a specific trend in the heights and intervals between successive peaks. First, the peak height appears to decrease linearly with time. Second, the interval is uneven and gradually shortened towards the end of decay. The observed pattern indeed suggests the presence of a certain regularity.

We discovered an interesting rule that satisfactorily determines the relative positions of the successive peaks. Suppose we assign the positions of the broad main peak (0th peak) and the following peaks, respectively, as indicated in Fig. 3. We find

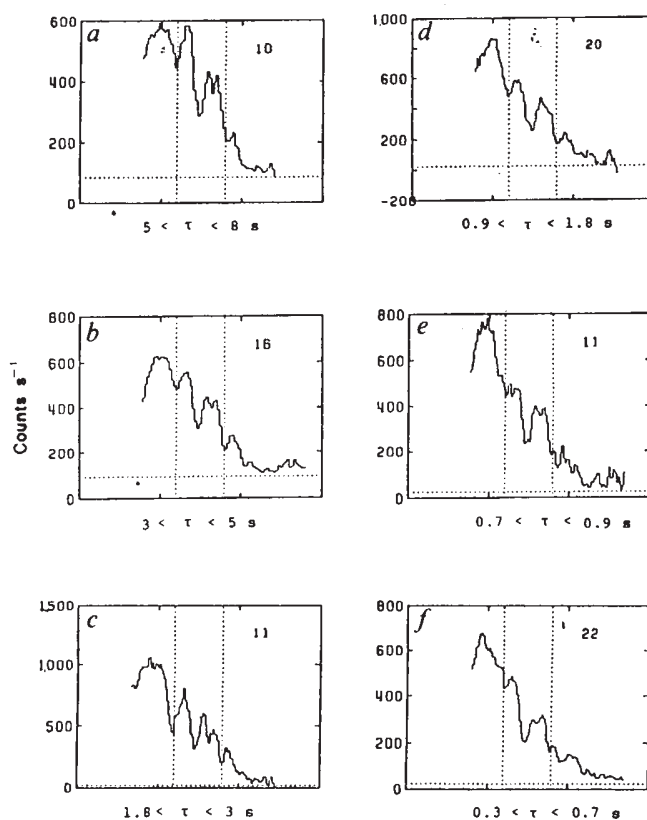


Fig. 2 Composite profiles of type II bursts constructed by normalizing the characteristic times, for six different ranges of τ . The number of bursts superimposed is indicated in each panel. *a-c* are from 1983 August data taken with a time resolution of 0.5 s, and *d-f* are from 1984 July data with a time resolution of 0.125 s.

that $(X_4 - X_2)/(X_2 - X_0) = (X_5 - X_3)/(X_3 - X_1) = \alpha$, with α approximately 0.57, where X_n is the time of the n th peak in units of the characteristic time. In other words, the successive peak positions are 'phased' in such a way that the intervals between the even-numbered peaks and those between the odd-

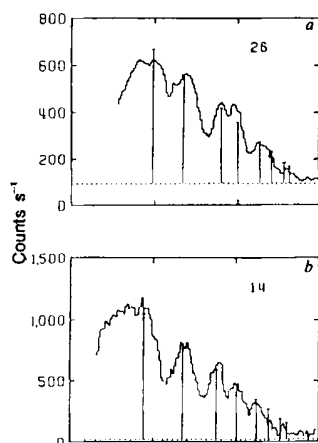


Fig. 3 Composite profile of the type II bursts of 1983 ($2.5 < \tau < 3.5$ s) (a) and 1984 ($2.0 < \tau < 3.5$ s) (b) activities. Vertical bars indicate the positions and heights of the peaks determined as described in the text.

numbered peaks respectively compose a geometric series with the same multiple α , although the peaks after the fourth are not always statistically significant. From the relative phase of the odd-numbered peaks with respect to the even-numbered ones, we define $(X_3 - X_1)/(X_2 - X_0) = \beta$, then $(X_1 - X_0)/(X_2 - X_0) = (1 - \beta)/(1 - \alpha)$, so that the geometric series of the even-numbered and odd-numbered peaks converge at the same point.

The same rule seems to hold for the heights of successive peaks. Namely, the successive peak heights, Y_n for the n th peak, roughly satisfy the following relations: $Y_2/Y_0 = Y_4/Y_2 = Y_3/Y_1 = Y_5/Y_3 = \alpha$ for the even-numbered and the odd-numbered peaks, with the same value of α as determined for the respective intervals; besides, $Y_1/Y_0 = \beta$. Thus, the common rule that applies to the intervals and heights makes the successive peaks appear to decrease linearly with time.

The above rule holds equally for the rapid bursts observed in 1983 as in 1984. There is a significant difference in the peak luminosity as well as the time-averaged luminosity between the activities of these two years. The bursts in the 1984 activity are on average roughly twice as luminous as those of 1983. The time-averaged luminosity during the 1984 activity is also about double that of 1983. It is important that, despite this difference, the values of α are the same in the two separate activities. However, a subtle difference exists in the relative phase of the odd-numbered peaks with respect to the even-numbered peaks. In the 1983 bursts, the odd-numbered peaks are shifted slightly earlier in phase than the corresponding peaks in the 1984 bursts; this is most noticeable in the smaller separation between the second and third peaks of the 1983 profile. The difference is expressed in terms of β which are 0.81 and 0.77 for the activities in 1983 and 1984, respectively.

If one assumes that the luminosity of a burst at a given time is determined by the instantaneous accretion rate, the above findings on the decay structure imply the presence of some complex flow-control mechanism. One might speculate that the structures are already prepared in the accretion disk (for example, regular concentric rings similar to the Saturn's rings). However, any model should explain the facts that the timescale-invariant profile is reproduced over a very wide range of burst duration while the peak luminosity (peak accretion rate) is independent of the burst duration and that the pattern is not influenced by the change of average accretion rate.

Search for a heavy neutrino in the β -decay of ^{35}S

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Recent experimental evidence¹ suggests that the neutrino (strictly, an antineutrino) emitted in the β -decay of tritium is a mixture of two mass eigen states $|\nu_1\rangle$ and $|\nu_2\rangle$ where $|\nu_e\rangle = \cos \theta |\nu_1\rangle + \sin \theta |\nu_2\rangle$ with $m_{\nu_1} < 50$ eV and $m_{\nu_2} = 17.1 \pm 0.2$ keV and $\sin^2 \theta = 0.03$. This conclusion is based on the observation of a 'kink' in the Fermi-Kurie plot^{2,3} of the electron energy spectrum at an energy 17.1 keV below the end point. Such an observation, if confirmed, will have far-reaching consequences in cosmology and particle physics^{4,5}. In the experiment reported here, a search was made for such an admixture in the β -decay of ^{35}S . We fail to confirm the presence of mixing of neutrinos with mass 17.1 keV and place an upper limit on such an admixture of 0.6% (90% confidence level).

The β -decay of ^{35}S has been studied extensively in magnetic spectrometers^{6,7}. ^{35}S decays with a half-life of 87.4 days to the ground state of ^{35}Cl ($3/2^+ \rightarrow 3/2^+$) with 100% branching with an end-point energy of 167.4 ± 0.2 keV (ref. 8). The transition is an allowed one, with a $\log ft = 5.0$. The linearity of the Fermi-Kurie plot has been established down to an energy of 20 keV (ref. 7). However, from the earlier data, particularly from the Fermi-Kurie plots which are usually available in the literature, it is not easy to discover any discrepancy like a 'kink' at around 150 keV, which is expected if a heavy neutrino admixture is present.

In the present experiment, a ^{35}S source in the form of BaSO_4 of less than $10 \mu\text{g cm}^{-2}$ thickness, was deposited onto a $90 \mu\text{g cm}^{-2}$ aluminized polypropylene foil. The β spectrum was measured with a shielded windowless liquid nitrogen-cooled Si(Li) detector of diameter 28 mm and thickness 3 mm fabricated in our laboratory. The detector had a resolution of 3.5 keV at 120 keV. The spectrometer was calibrated with conversion electron lines from ^{57}Co and $^{99\text{m}}\text{Tc}$ and γ -ray lines from $^{114\text{m}}\text{In}$ and ^{137}Cs . The conversion electron spectra were used to obtain the response function of the detector. The back-scattered fraction of electrons from the Si(Li) detector, in the energy range of

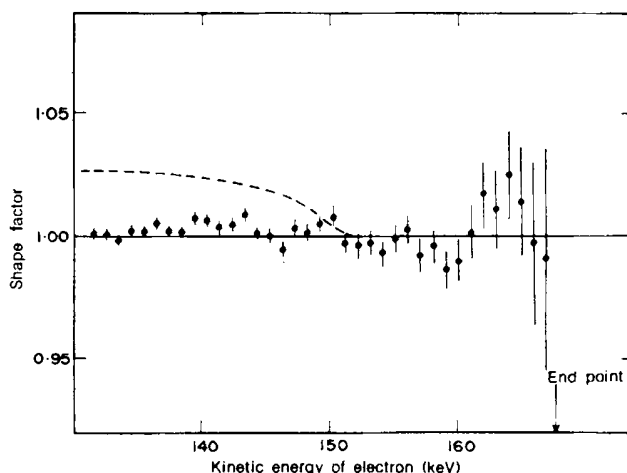


Fig. 1 The shape factor of the β -spectrum of ^{35}S as obtained in the present experiment ($\bullet$). Error bars, ± 1 s.d. Also shown (---) is the expected shape for a 3% branch in this β -decay where a 17-keV neutrino is emitted, with $\sin^2 \theta = 0.03$.

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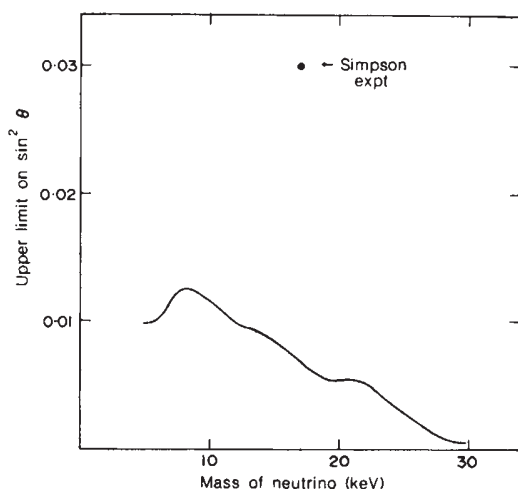


Fig. 2 Upper limit on $\sin^2 \theta$ at the 90% confidence level, obtained from the present experiment and shown as a function of the assumed neutrino mass. The region above the curve is excluded by this experiment. The single point shown at the top represents the result derived from Simpson's experiment¹.

130 keV, was determined to be 0.20 ± 0.02 at a source-detector distance of 8 cm, as used in the experiment. From a comparison of the electron and γ spectra of ^{99m}Tc , a dead layer of the order of 2 mg cm^{-2} was found to be present on the entrance window of the detector. Such a dead layer would not allow an accurate measurement of the β spectrum down to very low energies of $\sim 50 \text{ keV}$. However, this would not appreciably affect the shape of the spectra in the limited energy range 130–170 keV, hence we restricted our analysis to this energy region after correcting for the energy loss in the dead layer.

The counting rate was kept below 1,000 Hz and a time constant of $0.5 \mu\text{s}$ was used for the amplifier. Perspex collimators were used to block the electrons scattered from the chamber walls reaching the detector. These collimators also reduced the external bremsstrahlung produced from the walls of the vacuum chamber. The efficiency of the detector was determined from 75 keV to $\sim 1 \text{ MeV}$ using the internal conversion lines of the decay of ^{152}Eu . The measured efficiency was found to be constant to within $\pm 3\%$ in this energy region. By interpolation, the energy variation of efficiency can be estimated to be less than $\pm 0.2\%$ over the small energy range (130–170 keV) in which the data have been analysed. The measured β -spectrum was corrected for background and pile-up effects. The theoretical β spectrum for ^{35}S was calculated for $m_\nu = 0$ using the Fermi functions of Behrens and Janke⁹ and was folded with detector response function. The ratio of the experimental to the calculated spectrum normalized to 1 is the shape factor, and is shown in Fig. 1. The measured end point obtained is in agreement with the accepted value. If there is an admixture of heavy neutrinos of mass m with a mixing amplitude $\sin \theta$, such a shape factor $C(W)$ would have the form

$$C(W) = \cos^2 \theta + \sin^2 \theta \left[1 - \frac{m_\nu^2}{(W_0 - W)^2} \right]^{1/2} \text{ for } W < (W_0 - m_\nu)$$

$$C(W) = \cos^2 \theta \text{ for } (W_0 - m_\nu) < W < W_0$$

where W is the electron energy and W_0 the end-point energy. Also shown in Fig. 1 (broken curve) is the expected shape factor, with assumed values of $\sin^2 \theta = 0.03$ and $m_\nu = 17 \text{ keV}$ as reported by Simpson¹. It is clear from the figure that such a curve lies well outside our experimental limits, ruling out the existence of a mixture of a heavy neutrino of mass 17 keV and $\sin^2 \theta = 0.03$, in the β -decay of ^{35}S . In Fig. 2 the upper limits for $\sin^2 \theta$, at the 90% confidence level, derived from the above shape factor are shown as a function of the mass of the neutrino. It is clear that a heavy neutrino admixture of more than 1% does not exist in ^{35}S β -decay for the neutrino mass range 5–30 keV. Our results,

together with those of Schreckenbach *et al.*,¹⁰ do not suggest the presence of a heavy neutrino admixture above 1% for the neutrino mass range of 5–460 keV.

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Solid solution in plumbous potassium oxysilicate affected by interaction of a lone pair with bond pairs

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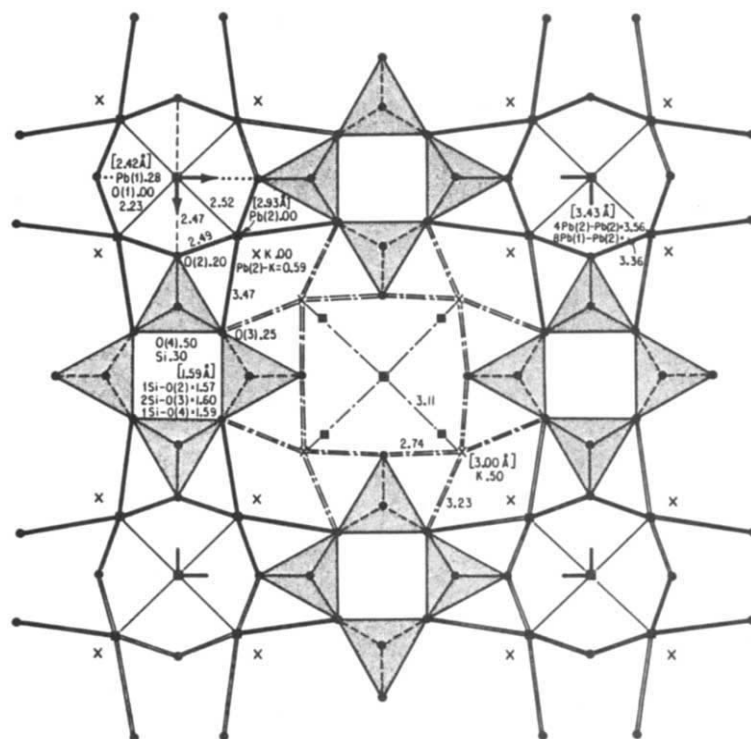
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During explorations of the chemical crystallography of lead silicates, the synthetic phase $\text{K}_2\text{Pb}_4\text{Si}_8\text{O}_{21}$ (here KPS), attracted our attention. Clear crystals when ground under acetone produced fine needles and fluffy aggregates which resembled chrysotile asbestos and had elicited some earlier interest¹. Evidence indicated tetragonal symmetry and despite several attempts no report of a successful structure solution has appeared. Indeed, the structure analysis proved to be a challenge, and revealed $[\text{Si}_8\text{O}_{20}]$ tubular columns and $[\text{Pb}_2\text{OPb}_4\text{O}_{24}]$ clusters, the latter consisting of six Pb^{2+} cations situated on the vertices of a compressed octahedron. A novel feature is the occurrence of disordered $(\text{Pb}^{2+}, \text{K}^+)$ as a 'solid solution', the $\text{Pb}-\text{K} = 0.59 \text{ \AA}$ separation being a consequence of the $6s^2 \text{Pb}^{2+}$ lone pair acting on circumjacent bond pairs. In our initial experience, best convergences yielded the reliability index $R \sim 0.20$. Unit cell parameters, space group and other structure cell criteria in Table 1 show good agreement between the present study and an earlier report² on KPS and isostructural $\text{Pb}_2\text{Pb}_4\text{Si}_6\text{Al}_2\text{O}_{21}$.

Our data set, on a crystal which was initially checked with assorted precession photographs and yielded condensed X-ray symbol $\text{I4}/***$, included 421 unique reflections for refinement. The data set was collected with an Enraf Nonius CAD-4 automated diffractometer utilizing graphite monochromator and $\text{MoK}\alpha_1$ radiation. All 3,326 reflections for $2\theta \leq 60^\circ$ were collected, and each was corrected for absorption based on an empirical ψ -scan, and symmetry-equivalent reflections were averaged. Unit cell dimensions (Table 1) were obtained by least-squares refinement of 25 reflections distributed throughout reciprocal space.

The final crystal structure was deciphered quite serenely. Three-dimensional Patterson maps quickly revealed the $\text{Pb}-\text{Pb}$ vectors, and attempts were made to locate the remaining $\text{Pb}-\text{X}$ ($\text{X} = \text{K}, \text{Si}, \text{O}$) vectors through models constructed in space group $\text{I4}/mmm$; and its subgroups $\text{I4}m$, $\text{I4}m2$ and $\text{I4}22$. A $\text{Pb}(1)$, $\text{Pb}(2)$ and K combination was introduced to a least-squares refinement, but the $\text{Pb}(2)-\text{K}$ site distance was

Fig. 1 Structure diagram of $\text{Pb}_2\text{O}(\text{Pb}_2\text{K}_2)[\text{Si}_8\text{O}_{20}]$ down $[001]$. The silicate tubes appear as stippled tetrahedra. Oxygens are located at solid disks, Pb atoms at squares and K atoms at crosses. Note O(1) superimposes on Pb(1) in this projection. Pb(2)-O bonds (solid) and Pb(1)-O bonds (dashed) are shown around the origin of the cell, K-O bonds (dot-dash) around body centre at $(\frac{1}{2}, \frac{1}{2}, \frac{1}{2})$. Individual bond distances (in Å, errors <0.01 Å) and polyhedral averages (in brackets) are shown adjacent to their geometric elements. Heights of atoms along c are given in fractional coordinates for their asymmetric unit.



only 0.6 Å. Surprisingly, $R = 0.10$. It then became clear that an apparent solid solution existed between Pb(2) and K. From that model, all other atoms could be located on a Fourier synthesis, which was based on space group $I4/mmm$. Continued coordinate parameter refinement followed by full-matrix anisotropic thermal vibration parameter refinement led to $R = 0.034$. Atom coordinate parameters from the final refinement appear in Table 2. We are forced to conclude that Pb(2) and K are coupled, both Pb(2) and K sites each half-populated about a centroid at $(\frac{1}{2}, \frac{1}{2}, 0)$. As we discuss below, these results are a consequence of an effect on solid solution from the interaction of $6s^2$ Pb^{2+} lone pair with neighbouring bond pairs. The compound is a plumbous potassium oxysilicate, more specifically $\text{Pb}_2\text{O}(\text{Pb}_2\text{K}_2)[\text{Si}_8\text{O}_{20}]$.

KPS is structurally related to some other well-defined arrangements. Figure 1 shows K-O, Pb-O bonds and $\frac{1}{8}[\text{Si}_8\text{O}_{20}]$ polyhedral linkages; Bond distances and polyhedral averages are also presented. Two distinct regions or fundamental building blocks can be discerned which, when isolated, show a direct relationship to some other structure. The first region is the $\frac{1}{8}[\text{Si}_8\text{O}_{20}]$ column or tube which runs parallel to the c -axial direction. Perhaps the simplest way to describe this silicate tube is by corner-linked condensation of $\frac{1}{8}[\text{Si}_2\text{O}_7]$ sorosilicate units, the tube generated by the 4_2 -screw operation parallel to the c -axis implicit in space group $I4/mmm$. This motif occurs in several distinct structure types, the simplest³ being narsarsukite, $\text{Na}_4\text{Ti}_2\text{O}_2[\text{Si}_8\text{O}_{20}]$. In the structure we report, the tube is more rigid, constrained by the presence of additional mirror planes which are normal to the a_1 and a_2 axial directions. The second region was an unexpected surprise. It is centred on O(1) at the

origin of the unit cell, and has local stoichiometry $\text{Pb}(1)_2\text{O}(\text{Pb}(2)_4\text{O}_{24})$, selecting the Pb end-member. A $\text{Pb}(1)_2\text{Pb}(2)_4$ cation cluster surrounds O(1), and is a compressed octahedron, with eight apical Pb(1)-Pb(2) = 3.36 Å and four equatorial Pb(2)-Pb(2) = 3.56 Å, with an average of Pb-Pb = 3.43 Å. From the individual polyhedral bond distances, it is evident that the $6s^2$ lone electron pairs in Pb^{2+} are situated on the outside of the $[\text{Pb}_6]$ octahedron in KPS. Note that the interatomic separation⁴ in Pb metal (face-centred cubic, f.c.c.) is 3.500 Å. This does not imply metallic Pb-Pb bonds in KPS, since the intervening anions and Pb-O bonds in the silicate must be included.

The Pb(2) and K sites, their half-occupancy the source of difficulty in structure solution, have separation K-Pb(2) = 0.59 Å. Decomposing the cluster into its polyhedral components, the $\text{Pb}(1)\text{O}(1)\text{O}(2)_4$ or $\text{Pb}(1)\text{O}_5$ defines a square pyramid of oxygen atoms coordinated to Pb(1). Alternatively, it can be described as an octahedron about Pb(1) with one vertex representing the lone electron pair (symbolized ψ) from $6s^2$ $\text{Pb}(1)^{2+}$. The Pb(1) atom is displaced 0.59 Å away from the equator of the $\text{Pb}(1)\text{O}_5\psi$ octahedron and towards the direction of the lone pair. Similar coordination polyhedra have been reported in several structures, although refinements which involve at least one heavy metal in an oxide matrix were usually inferior. A good example is the $\text{PbO}_4\psi$ polyhedron in the structure of tetragonal PbO or litharge, and refined from neutron diffraction data⁵. The shortest distance, Pb(1)-O(1) = 2.228(1) Å, opposes the assumed centroid of the lone pair. In litharge, the Pb atom is displaced 1.19 Å away from the equator and towards the direction of the lone pair. The average Pb-O distance in PbO_4 for litharge, a square pyramid with Pb as the apex, is 2.31 Å, significantly shorter than the 2.42 Å average for KPS. The short basal O-O = 2.80 Å separation in litharge contrasts with the tetragonal base distance O(2)-O(2) = 3.26 Å in KPS. These shorter distances in litharge are probably based on differences in oxygen coordination number about Pb(1), being 4 for litharge and 5 for KPS.

A large family of structures involves components Pb-X-O-H-Cl where $X = \text{Cu}^{2+}$, Mn^{2+} , Bi^{3+} , Sb^{3+} , As^{3+} , and so on, and many are based on similar structural principles. One well-refined structure⁶ includes diaboiteite, $\text{Pb}_2\text{Cu}(\text{OH})_4\text{Cl}_2$, with tetragonal space group $P4mm$ and one formula unit in the structure cell.

Table 1 Cell-structural data for $\text{Pb}_2\text{O}(\text{Pb}_2\text{K}_2)[\text{Si}_8\text{O}_{20}]$

	This study	Gibbs <i>et al.</i> ²	Narsarsukite ³
a (Å)	11.806(2)	11.816(6)	10.726(1)
c (Å)	7.913(2)	7.932(4)	7.947(1)
Z	2	2	2
Space group	$I4/mmm$	$I4/mmm$	$I4/m$
Specific gravity		4.40	2.78(1)
Density (g cm^{-3})	4.418		2.776
Formula	$\text{Pb}_2\text{O}(\text{Pb}_2\text{K}_2)[\text{Si}_8\text{O}_{20}]$		$\text{Na}_4\text{Ti}_2\text{O}_2[\text{Si}_8\text{O}_{20}]$

Table 2 $\text{Pb}_2\text{O}(\text{Pb}_2\text{K}_2)[\text{Si}_8\text{O}_{20}]$: atom coordinates and anisotropic thermal vibration ($\times 10^4$) parameters*

Atom	Cell	x	y	z	U_{11}	U_{22}	U_{33}	U_{23}	U_{13}	U_{12}
Pb(1)	4	0.0000	0.0000	0.2815(1)	405(4)	405(4)	93(3)	0	0	0
Pb(2)	$8 \times \frac{1}{2}$	0.1508(1)	0.1508(1)	0.0000	116(3)	116(3)	229(5)	0	0	-32(5)
K	$8 \times \frac{1}{2}$	0.1860(5)	0.1860(5)	0.0000	210(23)	210(23)	165(29)	0	0	57(30)
Si	16	0.3197(2)	0.0000	0.3006(3)	99(11)	298(14)	121(10)	0	-14(9)	0
O(1)	2	0.0000	0.0000	0.0000	645(119)	645(119)	148(106)	0	0	0
O(2)	16	0.2028(5)	0.0000	0.2057(7)	134(29)	277(34)	224(31)	0	-61(27)	0
O(3)	16	0.1113(5)	-0.3887(5)	0.2500	292(25)	292(25)	1,095(78)	-42(37)	42(37)	185(36)
O(4)	8	0.3031(12)	0.0000	0.5000	287(75)	2,970(273)	106(51)	0	0	0

The polyhedron of interest is $[\text{Pb}^{2+}\text{O}_4\text{Cl}_4]$ which is a distorted square antiprism. There exist $4\text{Pb}-\text{OH}=2.48$ and $4\text{Pb}-\text{Cl}=3.32$ Å averages. Electing $r=1.40$ Å for $^{[6]}\text{O}^{2-}$, $r=1.81$ Å for $^{[6]}\text{Cl}^-$ and $r=1.29$ Å for $^{[8]}\text{Pb}^{2+}$, the calculated averages are $\text{Pb}^{2+}-\text{O}=2.69$ Å and $\text{Pb}^{2+}-\text{Cl}=3.10$ Å, effective averages larger and smaller, respectively, than the experimental averages for diabolite. This was noted in the earlier study which further supports the interaction between the $6s^2$ Pb^{2+} lone pair with the enveloping bond pairs. It requires that the centroid of the lone electron pair is towards the direction of the plane of the Cl^- ions, foreshortening the opposing $\text{Pb}^{2+}-\text{OH}^-$ distances and lengthening the adjacent $\text{Pb}^{2+}-\text{Cl}^-$ distances with respect to the effective averages. An analogous relation can be found for $\text{Pb}(1)\text{O}_5\psi$ in KPS where $4\text{Pb}(1)-\text{O}(2)=2.47$ Å and $1\text{Pb}(1)-\text{O}(1)=2.23$ Å. If the centroid of the lone pair is postulated to oppose O(1) in KPS, then the short $\text{Pb}(1)-\text{O}(1)$ distance and the absence of $\text{Pb}^{2+}-\text{O}$ bonds on its opposing side can be understood. It is immediately apparent that the average for the Pb ion stripped of all its valence electrons, $^{[6]}\text{Pb}^{4+}-\text{O}=2.18$ Å from the same tables⁷, is not far removed from $\text{Pb}(1)-\text{O}(1)$ in KPS. Increase in average distances for coordination polyhedra of increasing order has been explained adequately elsewhere⁸. Similarly, displacement of a metal with a lone electron pair from the equator of a coordination polyhedron has electrostatic origins in the interaction implicit on a cation with a lone pair of electrons and the circumjacent bond pairs⁹.

The remaining polyhedron in the second region is a distorted monocapped cube, or $\text{Pb}(2)\text{O}_6$. The average distance is $\text{Pb}(2)-\text{O}=2.93$ Å, but the range in polyhedral distances is considerable, from $\text{Pb}(2)-\text{O}(2)=2.49$ Å to $\text{Pb}(2)-\text{O}(3)=3.47$ Å. We propose that the locus of the lone pair is on $\text{Pb}(2)$ opposite the $\text{Pb}(2)-\text{O}(1)$ bond, the considerable distortion in polyhedron and bond distances being due to the effect from interaction of a lone pair with the surrounding bond pairs. Further evidence for this interaction can be obtained from the same structure by selecting the site centroid of the KO_6 polyhedron associated with the purported $[\text{K}, \text{Pb}(2)]$ solid solution. The distances range from $\text{K}-\text{O}(2)=2.74$ Å to $\text{K}-\text{O}(3)=3.23$ Å with an average $\text{K}-\text{O}=3.00$ Å, a range of only 0.49 Å compared with a range of 0.98 Å for $\text{Pb}(2)-\text{O}$. This is a rare case where a cation with a lone pair of electrons in the valence shell can be directly compared with a cation where all valence electrons are stripped away in the same polyhedral environment which, in effect, constitutes a structural solid solution (compare with isostructural $\text{Pb}_2\text{Pb}_4\text{Si}_6\text{Al}_2\text{O}_{21}$).

Another feature is the similarity of the cluster in the second region to the structure of hyalotekite, a complex lead-barium-calcium-borosilicate-fluoride¹⁰. This compound is triclinic, but its cell is pseudo-tetragonal, with $a \sim 11.1$ Å, $c \sim 10.3$ Å and possesses two formula units in the cell. A weak structural relationship occurs between KPS and hyalotekite. Writing the formulae in sequence hints at this relationship.

KPS $\text{Pb}_2\text{O}(\text{Pb}_2\text{K}_2) [\text{Si}_8\text{O}_{20}]$ $\text{Pb}-\text{K}=0.59$ Å
 Hyalotekite $\text{Ca}_2\text{F}(\text{Pb}_2\text{Ba}_2) [\text{Be}_{0.5}\text{B}_2\text{Si}_{9.5}\text{O}_{28}]$ $\text{Pb}-\text{Ba}=0.46$ Å

In both structures, an apparent solid solution exists between a cation possessing a lone pair and a cation with a closed shell.

The KPS structure invites a simple experiment. The effect on a solid solution from interaction of a lone pair and neighbouring bond pairs with respect to temperature is unknown. Even the problem of solid solution between a cation possessing a lone pair (such as Pb^{2+}) and a cation stripped of all its valence electrons (such as K^{1+}) is not understood. We plan to study the crystal structure of KPS at elevated subsolidus temperatures to explore in more detail the bond distances and behaviour of the anisotropic thermal vibration ellipsoids through the thermal effect on the $\text{Pb}(2)-\text{K}$ site separation. Finally, since the lone electron pairs about Pb^{2+} are evidently circumjacent to the second aforementioned structural region and since the first region is a silicate tube, the curious appearance of fluffy fibrous material on grinding clear crystals suggests a pressure effect on the interaction between a lone pair and circumjacent bond pairs. Bond length, bond angle and solid solution dependencies on temperature and pressure would be most informative.

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An interhemispheric comparison of the concentrations of bromine compounds in the atmosphere

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At least seven organic bromine compounds have been positively identified in the atmosphere^{1,2} (CH_3Br , CH_2Br_2 , CHBr_3 , CH_2BrCl , CF_3Br , CF_2BrCl and $\text{C}_2\text{H}_4\text{Br}_2$) and others have been observed in coastal seawater samples ($\text{C}_2\text{H}_5\text{Br}$, $\text{C}_3\text{H}_7\text{Br}$ and CHBr_2Cl) (S. A. P. and R. A. Rasmussen, unpublished data). The atmospheric chemistry of bromine compounds has considerable consequence in the stratosphere, where bromine acts as a more efficient catalyst than chlorine in removing ozone^{3,4}. Several

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bromine compounds also fulfil an important role in the geochemical cycling of the element through the troposphere⁵. Here we present measurements of four bromine compounds (CF_3Br , CH_3Br , CH_2Br_2 and CHBr_3) over a large latitudinal range (40°N to 75°S). These data suggest that the major source of bromine in the atmosphere could be bromoform (CHBr_3), probably emitted from the ocean and with a short lifetime due to photolysis. Our data also suggest that the major emission of methyl bromide (CH_3Br), and hence most bromine entering the stratosphere, will be anthropogenic.

The specific sources of many bromine compounds are unknown, but are either anthropogenic or natural, with ocean chemistry and volcanic activity being obvious choices for the latter. The atmospheric lifetimes of the individual molecular species are likely to differ by many orders of magnitude, depending on the reaction rate with hydroxyl radicals or the photolytic destruction rate.

One way to categorize the source type and reactivity is to collect information on the latitudinal and longitudinal variation of the concentration of these compounds, in particular, to determine whether there is a substantial difference in concentration between Northern and Southern Hemispheric air samples. This is because the vast majority of anthropogenic sources are situated in the Northern Hemisphere and the interhemispheric transfer time is quite long (1–4 yr)^{6,7}.

Measurements of this type have been made at Harwell on air samples collected by the British Antarctic Survey (BAS) whose ships regularly traverse the Atlantic and Southern Oceans from about 50°N to 75°S on route from the UK to Antarctica. The samples were collected using a cryogenic trapping technique originally devised by Rasmussen⁸. Air was sampled on the bow of the BAS ships *RRS Bransfield* through a 10-ft length of steel pipe, which protruded out from the plating, and the sampling time was typically 10 min. The samples were only collected when the wind was on the bow or from a non-contaminating quarter, so the potential for contamination from the ship was very small. Typically 50 l of air were collected in each of the stainless steel sample bottles (volume 1.6 l), which were made to high specifications by Rasmussen. The properties of these containers with respect to contamination and decay of many trace gases have been studied extensively. Almost all trace gases were found to be stable at the high pressures (~ 450 p.s.i.) resulting from the sample collection process, when the air contained amounts of water vapour typical of surface samples. The wind direction was recorded at the time of sampling. No air mass trajectory calculations have been made due to difficulty in obtaining meteorological data.

In 1982, the ship sailed from Southampton to Cape Town, South Africa, passing the Canary and Ascension Islands along standard steamship routes and then to Antarctica. In 1983, the ship sailed from Southampton to Rio, Brazil, passing the Canary Islands and about 100 km east of the Cape Verde Islands along the steamship route and then on to Antarctica. The samples collected as far as Cape Town and Rio were air-freighted to London and analysed at Harwell within 6 weeks of collection. The samples collected farther south arrived back in England up to 6 months after collection. No marked difference in trace gas concentrations could be observed due to the different storage times.

The samples were analysed at Harwell for a large range of trace gases including halocarbons, hydrocarbons and sulphur compounds. In the case of the bromine compounds, which were the subject of this investigation, analysis was made using a highly sensitive gas chromatograph/mass spectrometer (GC/MS) combination developed especially for atmospheric trace gas measurements⁹. Before injection into the GC the air sample was enriched using a procedure very similar to that described previously¹⁰. This requires condensation of the gaseous air to liquid and removal of the permanent diluent gases under vacuum at liquid nitrogen temperature. The calibration procedure involved a static dilution technique which has been described in detail elsewhere¹¹. The detection limit was

~ 0.5 p.p.t.v. (parts per 10^{12} by volume) for CH_3Br , 0.2 p.p.t.v. for CH_2Br_2 and CF_3Br and ~ 0.1 p.p.t.v. for CHBr_3 . The precision of the analysis and sampling procedure was $\sim 10\%$ for all four compounds as the largest error arose in estimating the volume used in the analysis stage.

Data for the variation of concentration with latitude are shown in Fig. 1 for CH_3Br , CH_2Br_2 , CHBr_3 and CF_3Br . In the case of methyl bromide (CH_3Br), (Fig. 1a) there is a clear reduction in concentration between the two hemispheres, such that the average concentration in the Northern Hemisphere (NH) is 15.4 ± 1.9 p.p.t. and that in the Southern Hemisphere (SH) is 10.6 ± 0.9 p.p.t. There is also more scatter in the limited amount of Northern Hemisphere data while the variation in the southern data is very close to the limits of precision for the GC/MS system ($\sim 10\%$). A similar interhemispheric gradient has been observed previously¹² but the concentrations observed in both hemispheres were higher.

Methylene dibromide (CH_2Br_2) (Fig. 1b) also shows a marked hemispheric difference in concentration (NH = 2.70 ± 0.59 p.p.t.v., SH = 1.58 ± 0.26 p.p.t.v.) with again less variation in the Southern Hemisphere. The bromoform (CHBr_3) data (Fig. 1c) are much more scattered than either the CH_3Br or the CH_2Br_2 data. Although there is a tendency for the Northern Hemisphere concentrations to be higher, the mean interhemispheric difference is within the scatter of the data. The values for the mean and standard deviation are NH = 0.85 ± 0.44 p.p.t.v., when the value of 5.96 p.p.t.v. at 20°N is omitted, and SH = 0.58 ± 0.30 p.p.t.v. with the inclusion of the equatorial value of 0.88 p.p.t.v. These are both valid procedures since; first, the CHBr_3 concentration in the 20°N sample was elevated far above the average whereas none of the other trace gases measured showed such an elevation, and second, other trace gases such as ethane and some saturated hydrocarbons in the equatorial sample gave results typical of the Southern Hemisphere. There is no evidence that the high value at 20°N was associated with long-range transport of a plume of anthropogenic pollutants, because other trace gases indicative of pollution were present at levels similar to the average for the Northern Hemisphere samples. The slightly higher concentrations of CHBr_3 at 75°S in 1982 and 50°S in 1983 cannot be explained in terms of anthropogenic emissions. These data could mean that CHBr_3 has oceanic sources in both hemispheres and that it is more reactive in the atmosphere than are the mono- and di-bromo-substituted methanes. The CHBr_3 data contrast with those for CF_3Br (Fig. 1d) which show a very small gradient north and south on the 1982–83 voyage and a variability well within the limits of precision of the measurements (NH = 1.15 ± 0.05 p.p.t.v., SH = 1.06 ± 0.09 p.p.t.v.). CHBr_3 and CH_2Br_2 have been identified in coastal seawater (S. A. P. and R. A. Rasmussen, unpublished data).

The different variability in the concentration of the four molecules is associated with different source distributions and reactivity. The simplest behaviour is that of CF_3Br (CFC13BI) which is used as a flame retardant and is unlikely to have any natural sources. The molecule is mostly emitted into the atmosphere in the Northern Hemisphere and it has no significant tropospheric sinks. However, it is removed in the stratosphere by photolysis, as shown by measurements of its vertical profile up to 25 km (ref. 13). The small interhemispheric gradient is very similar to that of CFC 11 and 12, which have been growing in concentration at a rate close to $5\% \text{ yr}^{-1}$. A similar growth rate is therefore anticipated for CF_3Br . This compound was originally detected in the atmosphere in 1980 and its concentration then was estimated to be about 0.7 p.p.t.v. (ref. 10). Unfortunately, this was not a precise estimate and thus no absolute growth rate can be ascertained from our data at present.

CH_3Br is extensively used as a fumigant and annual production in 1972 was estimated to be about 300,000 tons, a quarter of which (75,000 tons) was probably emitted to the atmosphere³. It has also been proposed that the ocean is a major source, as in the case of methyl chloride and methyl iodide^{13,14}.

If it is assumed that CH_3Br is mostly removed from the

atmosphere by reaction with hydroxyl radicals in the troposphere, then the present data can be used to estimate the size of the annual input. A recently calculated value of the global average hydroxyl concentration is 4×10^5 molecules cm^{-3} (ref. 15); the atmospheric burden of CH_3Br is about 2.26×10^5 tons according to our data. These data require an annual input of 90,000 tons assuming removal only by hydroxyl radical chemistry with a rate constant of 3.2×10^{-14} cm^3 per molecule per s at 280 K (ref. 15). This is only slightly higher than the estimate³ of anthropogenic atmospheric emission made in 1972 of 75,000 tons, suggesting either that the ocean makes a relatively small contribution to the atmospheric burden of CH_3Br or that our rather simple chemistry is incorrect. An independent check on the chemistry can be made by comparing the methyl bromide levels in the two hemispheres. Assuming only a Northern Hemisphere source for CH_3Br which is transferred to the Southern Hemisphere with a relaxation time of 400 days, then the global average hydroxyl concentration required to account for the data is 3.6×10^5 molecules cm^{-3} , assuming all reactions take place at 280 K. The chemistry, therefore seems to be consistent and this leaves a problem with the oceanic source of CH_3Br . An estimate made recently¹² suggests that 300,000 tons of CH_3Br are emitted annually from the ocean. This would far outweigh the anthropogenic emission of CH_3Br and would argue for the globally-even distribution in concentration, observed for CH_3Cl (ref. 12), which has a similar removal rate from the atmosphere by hydroxyl radicals¹⁶. The global average hydroxyl concentration is an uncertain quantity and a larger total emission of methyl bromide into the atmosphere can be allowed, but the weight of evidence does not favour a large natural source.

Our interpretation of the data, therefore, suggests that the predominant source of CH_3Br in the atmosphere is anthropogenic rather than natural. This has important consequences for stratospheric ozone chemistry. It has been assumed previously that the stratospheric burden of bromine was mainly natural in origin and that any reduction in ozone produced by its presence was not a recent phenomenon¹⁰. This assumption is now questionable, and we suggest that stratospheric bromine chemistry should be examined more carefully in the context of possible effects of increased anthropogenic emissions.

The degree of variation in concentration suggests that both CH_2Br_2 and CHBr_3 are quite reactive in the troposphere, more so than CH_3Br and much more so than CF_3Br . They may have a mixture of sources since the CH_2Br_2 and CHBr_3 concentrations have been observed to increase during pollution incidents in the Arctic¹. Also, GC/MS studies of sea water containing different types of seaweed collected at a coastal site in southern England, show evidence for the presence of many different bromine compounds. These included $\text{C}_2\text{H}_5\text{Br}$, $\text{C}_3\text{H}_8\text{Br}$, CH_2BrCl , CHBr_2Cl and at least an order of magnitude greater quantities of CH_2Br_2 and CHBr_3 (S. A. P. and R. A. Rasmussen, unpublished data). It seems possible from the following calculations that CHBr_3 is the most important natural vector for gaseous bromine in the atmosphere and that CH_2Br_2 also has substantial natural sources. Their chemical lifetimes are unknown at present because of lack of published data either on rates of removal by hydroxyl radicals or on their photochemistry. However, the rates of reaction of the bromine compounds CH_3Br and $\text{BrCH}_2\text{CH}_2\text{Br}$ with hydroxyl radicals are very similar to those for their chlorine analogues. It is not unreasonable to assume, therefore, that to a first approximation, the rates for CH_2Br_2 and CHBr_3 are also similar to those for their chlorine analogues, for which published data exist: $k_{280\text{K}} = 1.128 \times 10^{-13}$ cm^3 per molecule per s for CH_2Cl_2 and 8.28×10^{-14} cm^3 per molecule per s for CHCl_3 (ref. 16). These rates should lead to a substantial interhemispheric gradient such as that observed for CH_2Cl_2 in our own unpublished data, where Southern Hemisphere values are only a quarter of the Northern Hemisphere values. The concentration distribution shown in Fig. 1 thus argues strongly for substantial sources of both CH_2Br_2 and CHBr_3 in the Southern Hemisphere, which must surely be natural rather than anthropogenic.

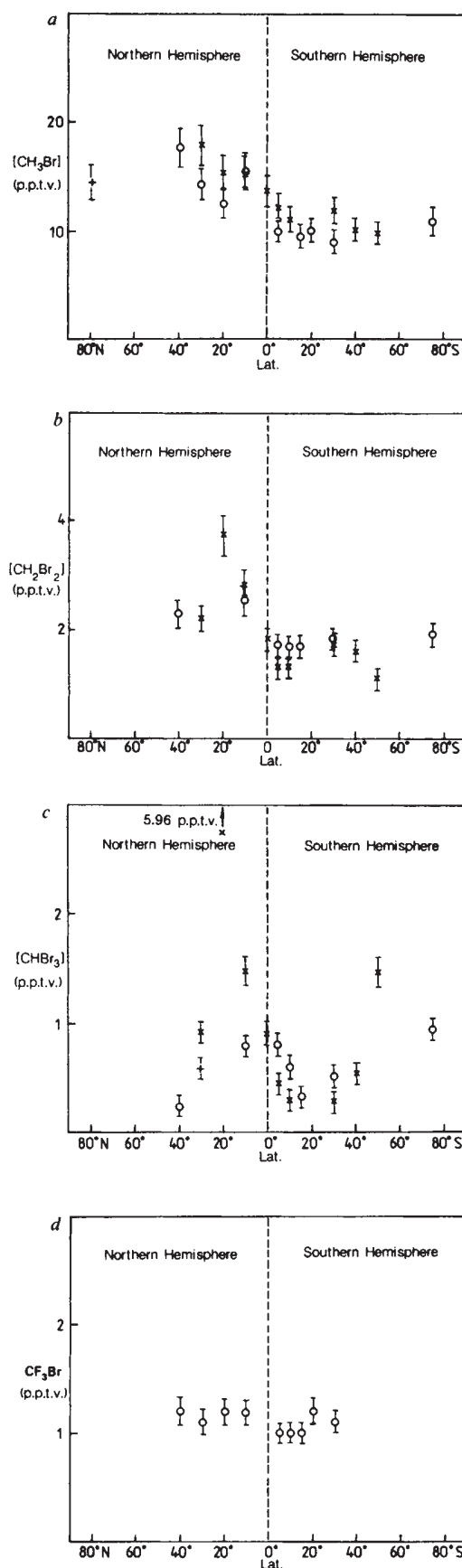


Fig. 1 Longitudinal transects of the concentrations of four bromine compounds: a, CH_3Br ; b, CH_2Br_2 ; c, CHBr_3 ; d, CF_3Br . $\circ$, 1982 voyage via Cape Town; $\times$, 1983 voyage via Rio; $+$, values collected on other experiments.

The case for natural sources of CHBr_3 is emphasized when a consideration is made of its potential atmosphere photochemistry. No published absorption spectrum could be found but some preliminary measurements were made at Harwell using a diode array spectrometer in conjunction with R. A. Cox. The absorption maximum occurs at a wavelength of 221 nm, where $\sigma = 5.51 \times 10^{-18} \text{ cm}^2$ per molecule and a significant overlap occurs in the region of tropospheric solar flux (305–320 nm). Assuming a quantum yield of unity, the atmospheric lifetime of CHBr_3 due to tropospheric photolysis could be as low as 2 weeks. This would require an annual emission in the region of 10^6 tons, which almost certainly exceeds the anthropogenic emissions many times over and makes CHBr_3 the most significant source of bromide released into the atmosphere. It is unlikely, though, that much CHBr_3 would penetrate into the stratosphere with a tropospheric lifetime this short.

Finally, there appear to be a substantial number of bromine compounds present in sea-surface waters that can find their way into the atmosphere. This indicates the presence of a rich bromine chemistry and mixed chlorobromo chemistry in the ocean that merit investigation in detail. The chemical behaviour of the bromine atoms released in both the troposphere and the stratosphere also needs study.

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Pyroxene solid solution in garnets included in diamond

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In what proved to be a temporary phase of trial mining of the Monastery Mine kimberlite pipe, South Africa, we recently had a brief opportunity to characterize the mineral inclusions in diamonds from this locality. The usefulness of such studies in elucidating mantle processes has already been demonstrated^{1–3}. We describe here garnets included in diamonds which have a unique range of compositions. They are interpreted as representing the first natural occurrence of garnet hosting a component of pyroxene in solid solution. Their discovery fulfils predictions based on successful laboratory experiments in which pyroxene was dissolved in garnet at very high pressures (50–180 kbar)^{4–6}. As this solid solution is accompanied by an increase in density, such results have both geophysical and chemical implications for the structure

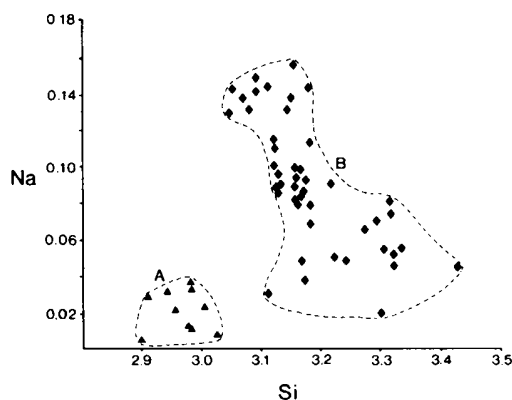


Fig. 1 Plot of atomic Na against atomic Si for 12 oxygens. Group A represents garnets of similar composition to eclogitic diamond inclusion garnets worldwide^{1–3,12–16}. Group B represents garnets interpreted to host a component of pyroxene in solid solution. Δ , Group A garnets; $\blacklozenge$, group B garnets.

of the mantle. In addition, the pressures inferred are higher than generally proposed for mantle minerals sampled by kimberlites. Recent work has demonstrated that at least some inclusion-bearing diamonds have been derived from Archaean lithosphere⁷. The Monastery garnets in diamonds may indicate that the old lithosphere was very thick^{8,9}. Alternatively, if they have an asthenospheric origin, they may prove particularly interesting because of their different source.

Monastery Mine is situated near the border between South Africa and Lesotho, close to the inferred eastern margin of the Kaapvaal Craton. It was an inviting target for a study of mineral inclusions in diamonds because it is geographically well separated from the established kimberlite diamond mines in southern Africa and because it is a kimberlite with an abundance of fresh xenoliths and xenocrysts of mantle rocks and minerals to which the inclusions and hence the diamonds might be related. The general geology of Monastery has been described elsewhere¹⁰.

In this study, 131 inclusion-bearing diamonds in the size range $-9+5$ (ref. 11) were selected from 4,600 carats of general production. The diamonds are <3 mm in largest dimension and average ~ 0.12 carats per stone. After careful visual inspection and rigorous cleaning, the inclusions were liberated from the diamonds by mechanical crushing in an enclosed steel cracker. The individual inclusions, usually between 75 and 250 μm in longest dimension were mounted in epoxy resin on glass slides and polished. Mineral compositions were determined by means of a Cameca Camebax microanalyser, using standards of essentially similar composition to the unknowns and an on-line ZAF correction procedure. Analyses are believed to be accurate to $\pm 1\%$ relative and have been checked at two independent microprobe laboratories.

The minerals found as inclusions in diamonds from the Monastery Mine kimberlite are almost entirely of an eclogitic paragenesis. Fifty-six eclogitic garnets have been analysed. Each analysis represents an average of at least five individual spots either on a single inclusion or several inclusions from the same diamond. Two distinct populations of eclogitic garnets are present in the diamonds (Fig. 1). One group (termed group A) consists of 10 garnets which have compositions similar to those described from diamonds worldwide^{1–3,12–16}. They have Na_2O concentrations which range from 0.04 to 0.25 wt% Na_2O , variable quantities of FeO , MgO and CaO typical of eclogitic garnets included in diamond, and SiO_2 and Al_2O_3 present in normal stoichiometric proportions, namely 39.0–42.2 wt% SiO_2 and 20.19–23.06 wt% Al_2O_3 (Fig. 2). The second group (group B) consists of 46 garnets that form a related suite of compositions which have never been reported before. Particularly extreme concentrations of Si and Al range to as high as 47.43 wt% SiO_2 and as low as 11.29 wt% Al_2O_3 (Fig. 2). Note that the low Al_2O_3 values are not supported by high Cr_2O_3 concentrations as occurs for peridotitic garnets, the maximum chrome concentration in

Table 1 Representative analyses of garnets included in diamonds

No.	1	2	3	4	5	6
Sample (Group)	A4-05 (A)	A4-03 (B)	B9-17 (B)	Al-20 (B)	Al-24 (B)	S-6
SiO ₂	41.76	42.08	43.45	45.43	47.43	46.11
TiO ₂	0.72	1.21	1.15	0.81	0.78	0.46
Al ₂ O ₃	22.45	18.32	16.30	14.82	11.29	15.64
Cr ₂ O ₃	0.29	0.01	0.06	0.06	0.20	0.43
FeO	9.29	14.74	12.99	9.77	10.51	8.04
MnO	0.23	0.27	0.27	0.29	0.19	—
MgO	20.60	10.31	13.48	18.20	22.05	20.88
CaO	4.31	11.89	11.93	9.27	7.11	8.16
Na ₂ O	0.08	1.08	0.64	0.58	0.33	—
Total	99.73	99.92	100.27	99.23	99.89	99.72
Cation proportions based on 12 oxygens						
Si	2.983	3.154	3.216	3.316	3.429	3.300
Ti	0.039	0.068	0.064	0.044	0.042	0.025
Al	1.890	1.618	1.422	1.275	0.962	1.320
Cr	0.016	—	0.004	0.003	0.011	0.024
Fe	0.555	0.924	0.804	0.596	0.635	0.481
Mn	0.014	0.017	0.017	0.018	0.012	—
Mg	2.193	1.152	1.487	1.980	2.376	2.227
Ca	0.330	0.955	0.946	0.725	0.551	0.626
Na	0.011	0.157	0.092	0.082	0.046	—
M*	3.089	3.188	3.329	3.382	3.608	3.334

Values given are for diamonds from Monastery Mine (analyses 1–5), while analysis 6 represents a garnet hosting a component of pyroxene in solid solution, synthesized experimentally at 146 kbar and 1,200 °C (ref. 6)

* M = Mg + Fe + Ca + Na.

these garnets being 0.37 wt% Cr₂O₃. Sodium can be present in extremely high concentrations, ranging from 0.14 to 1.03 wt% Na₂O (Fig. 2). Two garnets of similar composition to the group B garnets were reported from Jagersfontein by Tsai *et al.*¹⁴ but they did not comment on their unusual chemistry.

Representative analyses of both groups of garnets together with an analysis of an experimentally synthesized garnet⁶ are presented in Table 1. At first glance the extreme compositions in group B resemble amphibole compositions. However, samples spanning the compositional range of the garnets have been analysed by X-ray diffraction using a 57.4-mm Gandolfi camera and Co K α radiation. Extremely high-quality photographs were obtained and all unequivocally confirm a garnet structure for both groups of garnets. Cell parameters (*a*₀) calculated according to the equation

$$a_0^2 = (h^2 + k^2 + l^2)d_{hkl}^2$$

where *a*₀ is the unit cell parameter, *hkl* is the Miller index for a crystal plane, and *d* is the *d*-spacing of the crystal, and using the (10, 4, 0) reflection, range between 11.564 and 11.629 Å, which correlates well with cell parameters for pyrope, almandine and grossular (11.455, 11.53 and 11.85 Å respectively).

The pyroxene and garnet mineral families have closely related basic chemical formulae in that both possess cation to oxygen anion ratios of 2:3. Under appropriate conditions of pressure and temperature, small quantities of garnet may be taken into solid solution in pyroxenes^{17,18}. As pressure is increased, however, this solid solution breaks down into low-alumina pyroxene plus exsolved garnet¹⁹. Experimental evidence has demonstrated that the inverse of this reaction is also possible, namely the solution of pyroxene in garnet^{4–6,19,20}, and because of the large density difference involved, this reaction would also be expected to be strongly pressure dependent. Ringwood²⁰ demonstrated that as pressure increases, the solubility of pyroxene in garnet increases. Akaogi and Akimoto⁵ studied pyroxene–garnet solid solution equilibria over a wide range of temperatures and pressures in Mg- and Fe-rich systems. They found that the solubility of enstatite (MgSiO₃) in pyrope (Mg₃Al₂Si₃O₁₂) increases gradually from 60 to 140 kbar and then increases suddenly in the pressure range 140–175 kbar,

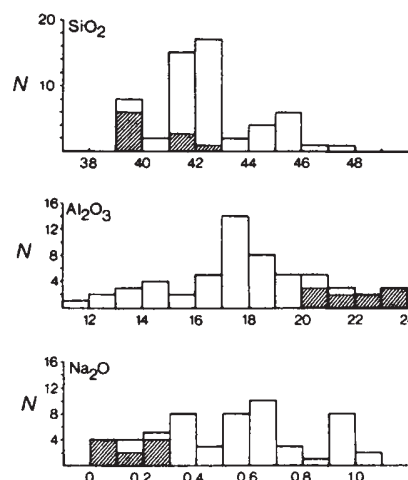


Fig. 2 Histogram of SiO₂, Al₂O₃ and Na₂O concentrations (wt%) in eclogitic diamond inclusion garnets from Monastery Mine. Shaded areas represent group A garnets (see Fig. 1 and text).

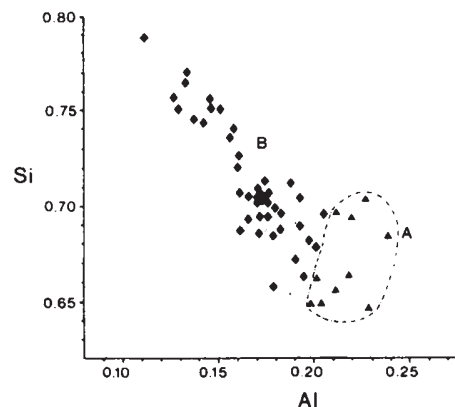


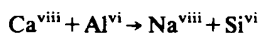
Fig. 3 Plot of Si (molecular proportion) against Al (molecular proportion) for eclogitic diamond inclusion garnets from Monastery Mine. Symbols as for Fig. 1.

resulting in the formation of a homogeneous garnet with a composition Mg₃(Al_{0.8}Mg_{0.6}Si_{0.6})Si₃O₁₂. Similar results were obtained for the ferrosilite–almandine system. The results of the experimental studies in simple systems have been verified and extended by Akaogi and Akimoto⁵, who successfully demonstrated a continuous increase in the solubility of pyroxene in garnet in a natural garnet lherzolite assemblage (PHN-1611) in the pressure range 45–205 kbar at temperatures of 1,050 and 1,200 °C. On the basis of these studies, Akaogi and Akimoto^{5,6} predicted that pyroxene would begin to dissolve in garnet at depths of 150 km in the upper mantle and that this would be manifested by an appreciable increase in the number of Si ions over the ideal value of 3 for 12 oxygens, indicating accommodation of Si ions in octahedral coordination.

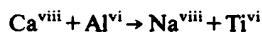
The group B diamond inclusion garnets at Monastery are interpreted in the light of the above experimental data. The number of Si ions for 12 oxygens in these garnets ranges from 3.046 to 3.429 (Fig. 1, Table 1), necessitating the existence of some Si in octahedral coordination. It is suggested that the group B garnets host a component of pyroxene in solid solution, an interpretation strongly supported by a comparison of the compositions of the group B garnets and the experimentally synthesized garnets⁶ (see Table 1, in particular analyses 4 and 6). Further evidence in support of this interpretation is the fact that the value of M (M = Mg + Fe + Ca + Na) in the group B garnets increases concomitantly with the increase of Si from 3.0 (see Table 1). Although M is not a good indicator of the presence of pyroxene in solid solution in garnet compared with Si, an increase of both M and Si is required to form garnet solid solution with pyroxene components (since pyroxene ~ Si + M).

Note that the Ca-rich garnets tend to have lower Si contents than the magnesian garnets, indicating that they have less pyroxene in solid solution. This is consistent with the data of Akaogi and Akimoto⁶, who found that systematically higher pressures are required for the solution of pyroxene in garnet in Ca-rich systems than in Fe- and Mg-rich systems. In addition, the Fe-Ca-rich group B garnets show extreme enrichment in Na and lesser depletions of Al than the magnesian group B garnets. This may be due to a similar effect to that described for inclusions in diamonds from Orapa³, where the composition of the pyroxene molecule in equilibrium with garnet changes with garnet composition. The major change is an increase in the jadeite molecule at the expense of diopside as iron and calcium in garnet increases. Solid solution of such pyroxenes in eclogitic garnets would be expected to produce the trends noted above.

Sobolev and Lavrent'ev²¹ suggested that elevated concentrations of Na in garnet may be correlated with high pressure. They proposed that Na enters the garnet structure according to the equation



thereby requiring some Si to be present in octahedral coordination. Thompson²² reported 0.06–0.57 wt% P_2O_5 in garnets synthesized from anhydrous basaltic melts at 18–45 kbar and 1,200–1,450 °C and proposed a coupled substitution between Na–P and Ca–Si. He points out that since the sum of atomic radii of Na and P is less than that of Ca and Si, the substitution of an Na–P couple into the garnet structure would be enhanced by high pressure. Bishop *et al.*²³ favour the idea that Na in garnets from peridotites and eclogites can be explained by substitutions of the type



without the need to invoke octahedral Si.

The group B garnets have been carefully analysed for P and found to have <0.01 wt% P_2O_5 . The coupled substitution between Na–P and Ca–Si is thus not feasible in this case. The coupled substitution between Ca–Al and Na–Si proposed by Sobolev and Lavrent'ev²¹ is also not possible, because of the existence of a negative correlation between Si and Na in these garnets (Fig. 1). The excellent negative correlation observed between Si and Al (Fig. 3), however, probably indicates that the substitutional scheme proposed by Akaogi and Akimoto⁶ to account for the compositions of their experimentally synthesized garnets (for example, Table 1, analysis 6) is also applicable to these garnets. The scheme involves the substitution of two Al^{3+} ions in the octahedral sites of the garnet structure by M^{2+} and Si^{4+} ions from the pyroxene structure ($\text{M}^{2+} = \text{Mg}^{2+}$, Fe^{2+} and Ca^{2+}). Na may be accommodated in the eight-coordinated dodecahedral site, as suggested by Sobolev and Lavrent'ev²¹.

The highest reported sodium value in an eclogitic diamond inclusion garnet was until recently 0.26 wt% Na_2O (ref. 21), but Hall and Smith²⁴ have reported Na-rich eclogitic garnets from Argyle, the highest concentration being 0.52 wt% Na_2O . Although they also failed to find a correlation between Na, Ti and P, appreciable P_2O_5 was detected and there is no evidence in the published analyses for excess silica or a deficiency of aluminium. Their garnets do not, therefore, appear to be like those described here.

The discovery of garnets from the upper mantle hosting pyroxene in solid solution and having Si in octahedral coordination is extremely important in view of current petrological modelling of the mantle. A controversial issue is whether the eclogite to garnetite transition is responsible for the 400-km seismic discontinuity^{25–27}. Our data show that the dissolution of pyroxene in garnet does occur in the upper mantle and, therefore, strengthens the evidence for the feasibility of the garnetite transition.

It is generally believed that diamonds have crystallized at depths >150 km (ref. 1), but the temperatures of equilibration calculated for coexisting diamond inclusion pairs^{1–3,12,13,24} are generally so low that origins deeper than 200 km have not been suggested. Palaeogeothermal reconstructions^{28,29} based on xenolith mineral compositions from kimberlite terminate at similar depths, which could mark the lithosphere–asthenosphere boundary. The group B garnets range to compositions showing extensive pyroxene solid solution in garnet, which is not seen in other mantle-derived garnets. As this solid solution is a predominantly pressure-dependent reaction, it seems likely that the group B garnets have an extremely deep origin. Based on experiments by Akaogi and Akimoto⁶ on a natural garnet lherzolite, pressures well above 100 kbar are possibly required; for example, garnet 6 in Table 1 was synthesized at 146 kbar. Note, however, that the pressure required to allow pyroxene solubility in garnet is substantially lower in Fe-rich systems than in Mg- and Ca-rich systems⁶. As the garnets under discussion are reasonably Fe-rich, the pressures required for their synthesis should not be as high as those required to produce pyroxene–garnet solid solution in the natural garnet lherzolite⁶. Therefore, no restricted range of likely depths of origin can be justified on the available evidence. Note that as a group 1 kimberlite³⁰ the Monastery diatreme has an asthenospheric signature, and the diamonds with group B inclusions are therefore not constrained to a lithospheric origin in the absence of any knowledge of their isotopic characteristics.

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Forest changes in the Amazon Basin during the last glacial maximum

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We report here the discovery of forest beds in the Amazon Basin radiocarbon dated to the Wisconsin–Würm glaciation. These are the first dates from the last glaciation determined on Amazonian samples. The locality is in Ecuador, at 1,100 m elevation, near the upper limit of the present Amazonian rainforest. Both pollen spectra and *Podocarpus* wood in the deposits suggest that vegetation comparable to the present Andean forest grew at least 700 m lower than it does now, suggesting a temperature depression of at least 4.5 °C for the Amazon lowlands.

The forest beds are exposed along road cuts at two sites near Mera (1°28' S, 78°6' W, elevation 1,100 m) in Oriente Province, Ecuador (Fig. 1). The area has a mean annual temperature of 20.8 °C and precipitation of >4,800 mm, making it one of the wettest places in the Amazon Basin¹. The present vegetation is tropical forest of high diversity, at the transition between lower montane rainforest and the true lowland rainforest which begins at ~800 m elevation².

At the first exposure, an organic bed ~2 m thick is overlain by ~12 m of largely inorganic deposit (Fig. 2). Unsorted angular boulders both above and below the organic layer are embedded in a clastic matrix, suggesting lahars, though this must be confirmed by further field work. A darker lithological unit, possibly organic, overlies the upper debris flow and is in turn overlain by a fluvial sequence of clast-supported gravel and sand.

The organic bed has woody twigs or roots up to 10 cm in diameter running through it, has a loss-on-ignition ranging from 30 to 64%, and has high pollen concentrations. It thus appears to be a marsh or soil deposit that accumulated before burial under a debris flow. Two wood samples were collected *in situ* from the forest bed (samples 1 and 5) and sediment samples were collected at 0.3-m intervals for pollen analysis. Wood sample 5 yielded a radiocarbon date of $33,520 \pm 1,010$ yr BP (B-9618).

The second exposure lies about 3 km east of the first. The road-cut section is ~20 m high here and consists of deeply weathered fluvial sand at the base, overlain unconformably by a clast-supported gravel with rounded boulders. The forest bed, ~2 m thick, lies above the gravel and is overlain by fluvial sand. It could not be reached to obtain samples, but slumped blocks containing parts of tree trunks and stumps up to 30 cm in diameter at the foot of the section had apparently fallen from the fossil forest bed. This wood was embedded in a clay-supported matrix of angular pebbles. Wood samples numbers 2–4 were taken from the slumped blocks. Sample 4 has a radiocarbon age of $26,530 \pm 270$ yr BP (B-10170).

Wood samples from both forest beds were submitted to R. B. Miller (USDA Forest Products Laboratory, Madison) for identification. Wood samples 1, 2 and 4 are soft wood from conifers, although poor preservation precludes more precise identification. *Podocarpus* is the only conifer genus now occurring naturally in Ecuador³. *Pinus*, the conifer next nearest to Ecuador, grows no nearer than Nicaragua, at least 1,000 km away⁴. It is therefore reasonable to conclude that wood samples 1, 2 and 4 are of *Podocarpus*. There are indications that sample 1, from the first site, is of a different species from the 6,000-yr younger samples 2 and 4 of the second site.

Wood samples 3 and 5 are of hardwoods, sample 5 possibly of the genus *Tovomita* (Guttiferae), a genus with both Andean and Amazonian species in the modern flora of Ecuador.

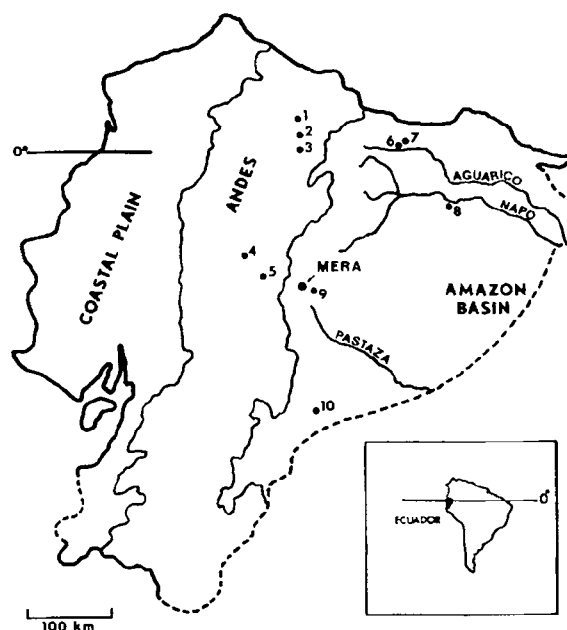


Fig. 1 Regions of Ecuador showing the Mera site of the Pleistocene forest beds and of the suite of surface samples. 1, Lake Conru; 2, Lake San Marcos; 3, Yaguarcocha; 4, Lake Yambo; 5, Rum Tum; 6, Lake Sta. Cecilia; 7, Lake Agrio; 8, Añangucocha; 9, Puyo Bog; 10, Lake Kumpak^a.



Fig. 2 Mera forest bed: first exposure. Arrows mark edges of polleniferous organic layer containing woody parts of *Podocarpus* dated at $33,520 \pm 1,010$ yr BP.

Pollen concentration (calculated by the exotic pollen technique) of 660,000 grains per cm^3 in a sample of the organic bed at site 1 is almost an order of magnitude higher than concentrations typical of the Andean and Amazonian lake samples of Fig. 1. Table 1 compares the percentage pollen spectra from this sample with 10 surface or recent spectra from Ecuador, 5 from the Andes and 5 from the Amazon forest. The sample from Puyo

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Table 1 Pollen from Mera forest bed compared with modern pollen spectra

	Conru (2,800 m)	Yambo (2,600 m)	Yaguarcocha (2,210 m)	San Marcos (3,400 m)	Rum Tum (2,392 m)	Kumpak ^a (700 m)	Añangucocha (230 m)	Puyo Bog (953 m)	Lago Agrio (330 m)	Sta Cecilia (330 m)	Mera (1,100 m)
<i>Podocarpus</i>		0.3	0.4								0.4
Palmae				0.6				0.3	0.6	1.0	
<i>Iriartea</i>					0.3	2.3	2.4	0.3	13.3	9.3	1.2
<i>Cecropia</i>	0.7	2.3	0.4			43.1	63.4	43.3	39.3	49.8	0.8
<i>Ficus</i>						3.4		1.2	7.2	1.0	
Urticaceae-Moraceae	5.2	10.6	4.4	1.7	2.0	10.4	4.8	17.3	14.5	14.1	4.0
Melastomataceae	1.4	3.0	1.6	1.0	1.7	1.9		1.5	0.9	0.3	7.6
<i>Trema</i>		0.7		0.7		0.4		1.2	2.3	2.3	
<i>Piper</i>						1.9	1.6	0.6	3.5	0.7	
<i>Alnus</i>	2.1	1.0	0.4	0.3							4.8
<i>Hedyosmum</i>	0.3			0.3							10.0
<i>Acalypha</i>						13.7		0.9	0.9	2.3	
Gramineae	36.8	27.3	14.0	37.7	70.0	1.5		6.3			0.8
Compositae	1.7	2.3	2.4	5.7	4.3		0.8	2.7	0.3	1.0	0.8
<i>Plantago</i>	3.8	2.0				0.4					
<i>Rumex</i>	8.3	1.0	1.6								
Chen-Am	1.7	8.7	7.8		0.3	0.4	0.8				
Cyperaceae	27.4	5.7	57.8	2.7	1.3		1.6	1.5			
Monolete	0.7	2.0	0.8	11.3	2.3	2.7	10.6	2.7	1.7	1.6	13.6
Trilete	0.9	3.0	2.4	5.0	0.3	2.3		1.3		2.3	2.8
'Others'	12.5	31.9	18.7	29.6	11.6	21.0	27.3	19.5	18.9	14.3	53.2

Sites shown on Fig. 1. Añangucocha sample is from surface pinches of moist forest soil; Puyo Bog is surface organic matter of a tropical swamp; Rum Tum is mud from a small pond and Yaguarcocha is from just below the settlement layer of a sediment core to yield pollen from the Interandean Plateau before human disturbance. Remaining samples are from mud-water interfaces of lakes. We distinguish >200 pollen taxa in Amazonian fossil pollen samples, although many of these cannot yet be given taxonomic names. We have included as 'others' many identifiable, but infrequent pollen types as well as a considerable number of tricolporate and tricolpate taxa yet to be identified. We have also combined subdivisions of other taxa like Moraceae and Urticaceae.

Bog is from within 5 km of site 1 in the same forest type. Table 1 shows that the pollen rain falling at Mera 33,000 yr ago is comparable to pollen of high elevations in the modern Andes and not to pollen emanating from the lowland rainforest like that falling near Mera today. The inclusion of significant percentages of the Andean taxa *Alnus* and *Hedyosmum* is particularly striking, as is the virtual absence of the *Cecropia* found at high percentages in every sample we have yet examined from Holocene and surface samples from Ecuadorian Amazonia, including the immediate vicinity of Puyo.

The Pleistocene pollen assemblage is Andean, although not closely comparable to pollen from any modern facies of the Andean forests. For instance, the taxa Melastomataceae, monolete fern spores, and *Hedyosmum* are in proportions not closely matched by the Andean spectra available. One explanation of an unusual pollen assortment would be pollen transport and redeposition by streams mixing pollen from different elevations, but the very high pollen concentration and organic matrix of the deposit make it unlikely that the beds represent stream deposits. This conclusion is supported by the presence of *Podocarpus* megafossils (twigs or roots) embedded in the polleniferous organic matrix of site 1. The *Podocarpus* megafossils are unlikely to have been redeposited in a richly polleniferous organic sediment by rapid mountain streams. The data thus suggest that Andean forest including *Podocarpus* grew at 1,200 m elevation at times during the last glacial cycle.

Podocarpus typically grows today in the Andean forest between the 2,000- and 3,500-m contours in Ecuador and Colombia^{2,3,5,6}. Four species occur naturally in Ecuador: *P. montanus* (including *P. montanus* var. *densifolius*), *P. oleifolius*, *P. glomeratus* and *P. sprucei*. Altitudinal ranges given in the literature are 2,000–2,800 m for *P. oleifolius*, 2,500–4,000 m for *P. glomeratus* and 2,000–3,000 m for the whole section of *Stachycarpus*^{3,5}. These reports suggest that 2,000 m is about the lowest altitude at which populations of *Podocarpus* are to be expected. The lowest confirmed modern elevation in the records of the Fundacio Natura of Ecuador is 1,800 m.

The late Pleistocene populations of *Podocarpus* grew at least 700 m below the lowest modern populations. If, as seems likely for mountain conifers, the altitudinal ranges are temperature dependent, we can calculate the necessary temperature depression. Assuming a lapse rate of 0.65°C per 100 m, the minimum lowering of mean annual temperature is 4.5°C. As the pollen data suggest extensive montane forests at this eleva-

tion, the actual lower limit of *Podocarpus* was certainly below 1,100 m and the actual temperature depression >4.5°C. This conclusion is compatible with earlier estimates of a temperature depression of 6°C on the altiplano of Colombia⁶. These new data, however, are the first to demonstrate temperature depressions of this magnitude in the Amazon lowlands themselves.

Note that both our radiocarbon dates fall within various definitions of a mid-Wisconsin interstade⁷, suggesting that forest and temperature depressions at full glacial times were even larger than our calculations suggest. There are no data which could establish the extent to which an interstadial was expressed along the American Equator, although an episode of glacial advance younger than 25,000 yr ago has been documented from the Ecuadorian Andes⁸. It is likely that the displacement of vegetation and climate during the glacial maximum at 18 K was even larger than that suggested by the Mera data.

These data are of particular interest in view of the hypothesis that much of the Amazon Basin was arid during times of glacial maxima, with the rainforest reduced to several large refugia in regions of highest modern rainfall^{9–11}. Prominent among suggested refugia is the region between the Napo and Pastaza rivers in which the Mera sites lie, since this is the Amazonian region of highest contemporary rainfall. Our pollen data show that at least part of the territory of this supposed refugium in fact supported forest comparable to modern, montane Andean forest from 33,000–26,000 yr BP in Wisconsin–Würm times.

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Calcium gradients in single smooth muscle cells revealed by the digital imaging microscope using Fura-2

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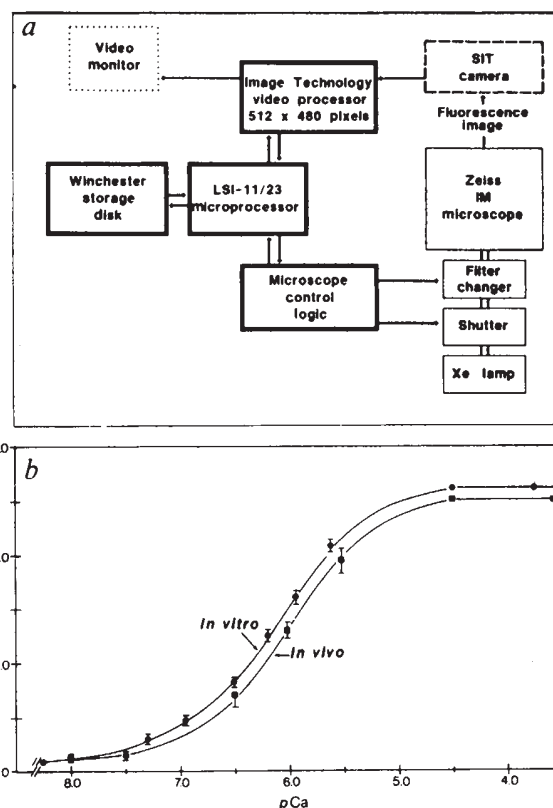
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Calcium is believed to control a variety of cellular processes, often with a high degree of spatial and temporal precision. For a cell to use Ca^{2+} in this manner, mechanisms must exist for controlling the ion in a localized fashion. We have now gained insight into such mechanisms from studies which measured Ca^{2+} in single living cells with high resolution using a digital imaging microscope and the highly fluorescent Ca^{2+} -sensitive dye, Fura-2. Levels of Ca^{2+} in the cytoplasm, nucleus and sarcoplasmic reticulum (SR) are clearly different. Free $[\text{Ca}^{2+}]$ in the nucleus and SR was greater than in the cytoplasm and these gradients were abolished by Ca^{2+} ionophores. When external Ca^{2+} was raised above normal in the absence of ionophores, free cytoplasmic Ca^{2+} increased but nuclear Ca^{2+} did not. Thus, nuclear $[\text{Ca}^{2+}]$ appears to be regulated independently of cytoplasmic $[\text{Ca}^{2+}]$ by gating mechanisms in the nuclear envelope. The observed regulation of intranuclear Ca^{2+} in these

contractile cells may thus be seen as a way to prevent fluctuation in Ca^{2+} -linked nuclear processes during the rise in cytoplasmic $[\text{Ca}^{2+}]$ which triggers contraction. The approach described here offers the opportunity of following changes in Ca^{2+} in cellular compartments in response to a wide range of stimuli, allowing new insights into the role of local changes in Ca^{2+} in the regulation of cell function.

The resting Ca^{2+} distribution in smooth muscle was determined using a digital imaging microscope to measure the fluorescence of enzymatically disaggregated toad (*Bufo marinus*) stomach cells loaded with Fura-2. This cell type has been studied in detail (for review see ref. 3), previous work having revealed that, like other types of smooth muscle, these cells use Ca^{2+} from different sources for different stimuli⁴⁻⁶. The recording system (shown schematically in Fig. 1a) was developed to detect light within a single cell with extremely high accuracy, sensitivity and resolution, so that a very small number of molecules could be detected in a limited region of a cell⁷. $[\text{Ca}^{2+}]$ at each point in a cell was determined by measuring the fluorescence intensity (500 nm, emission) with excitation at 340 nm and at 380 nm² (Fig. 2a, b). The 340-nm image (Fig. 2a) showed large regional inhomogeneities in fluorescence intensity which reflected both Ca^{2+} and dye distribution, and which were also influenced by cell geometry. Division of this image, on a pixel-by-pixel basis, by that obtained with 380 nm (Fig. 2b) resulted in a ratio image (Fig. 2c) in which variations in fluorescence intensity within the cell were indicative² of spatial variation in $[\text{Ca}^{2+}]$. The nuclear and sub-plasmalemmal fluorescence detail,

Fig. 1 Acquisition of Fura-2 cellular fluorescence images and determination of cellular $[\text{Ca}^{2+}]$. **a**, Schematic illustration of digital imaging microscope used to acquire and process images of cellular Fura-2 fluorescence. Fluorescence of Fura-2-loaded single smooth muscle cells was imaged using a Zeiss Ultrafluor 100 $\times$, NA = 1.25 objective and an inverted microscope (Zeiss IM) equipped for epifluorescence. Video images were obtained using a silicon-intensified target camera (Dage MTI, model 66), with the output digitized to a resolution of 512 $\times$ 480 pixels by an Image Technology video analyser with each point in the image being assigned a value from 0 to 255 depending on intensity. The shutter/filter changer were obtained from Digital Microscope Accessories Co. The optics and digital imaging system provide better than 0.25 μm resolution in a given image plane. However, the large depth of focus of the fluorescent microscope results in information from a much greater depth than this being present in a given image plane⁷. For example, light from a point source 2 μm either above or below a given specimen plane will be attenuated to only 30% at the position in the image corresponding to the point source. Images were stored on a high-capacity Winchester disk (20 megabyte capacity) and digitally analysed using a PDP-11/23 microcomputer. Multiple video frames were sequentially added to memory to increase the signal-to-noise ratio in the averaged image. To correct for spatial variations in dark current of the camera, averaged images, obtained without input to the camera, were subtracted from the images on a pixel-by-pixel basis. To correct for spatial variations in the intensity of the excitation source, as well as spatial variations in camera gain, the image was multiplied pixel-by-pixel with a 'system gain normalization' map. This map was constructed by obtaining an image of a uniform fluorescent field (a well-mixed solution of Fura-2 free acid), with intensities at each point being divided by the mean fluorescence intensity so that the gain normalization did not change the mean grey level of an image. Images of Fura-2 fluorescence at 500 nm emission were obtained with 340 and 380 nm excitation wavelengths using filters from Dittic Optics Inc. (Hudson, Massachusetts) (emission filter, 500 nm, Halfwidth, 20 nm; excitation filters, 340 nm, Halfwidth, 10 nm; 380 nm, Halfwidth, 10 nm). The ratio image ($R_{340/380}$) was achieved from the division of the 340-nm image by the 380 nm image on a pixel-per-pixel basis. A fluorescence intensity threshold was set before the division to exclude non-cellular regions from the determination of the ratio image. We have not attempted to interpret information in pixels immediately adjacent to the cell edge due to a low signal-to-noise ratio, and the extreme sensitivity of the ratio image at these points to small mechanical and/or electronic shift between the two images. All images were photographed using a Celtic Technology video/filter recorder. **b**, Relationship between Fura-2 fluorescence ratio and $[\text{Ca}^{2+}]$. *In vitro* calibration: smooth muscle cells were loaded with Fura-2 (Fura-2/AM) for 90 min. The external loading solution (amphibian Krebs Ringer or a similar HEPES-based buffer) was removed by gentle centrifugation (~200 r.p.m.) and replaced by a solution very like the composition of the intracellular milieu: (mM) K^+ , 100; Na^+ , 30; Mg^{2+} , 1; ATP, 5; Balanced Electrolyte Solution, 20. Small aliquots of cells (300 μl) were each exposed to different external $[\text{Ca}^{2+}]$. The pCa of the bathing solution was controlled by varying the ratio of Ca-EGTA/EGTA, and was determined by potentiometric titration of free and total (~10 mM) EGTA concentrations by CaCl_2 and CdSO_4 , respectively^{25,26}. This solution also contained 1–2 μM ionomycin to allow equilibration of intra- and extracellular Ca (ref. 6). Higher concentrations of ionomycin (3–5 μM) can completely remove the external cell membrane in this cell type. Fluorescence ratios (340/380) were obtained from several individual cells from each aliquot. Absolute measured ratios are displayed as a function of the calculated pCa of the external solution. $[\text{Ca}^{2+}]$ determined using this calibration curve coincided closely ($\pm 5\%$) with values determined using the standard calibration equation (Fig. 3 legend). Possible differences in dye properties due to binding to highly immobile cellular sites were investigated by determining the fluorescence emission anisotropy ($r = [(I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})]$)²⁷ under the microscope (380 nm excitation). Fluorescence anisotropy values within different cellular compartments (cytoplasm, 0.08 ± 0.01 , $n = 11$; nucleus, 0.08 ± 0.02 , $n = 6$) were not significantly different from those produced by free dye in solution (0.05 ± 0.01 , $n = 8$). Much higher anisotropies (up to 0.34) are observed when the dye is embedded in highly viscous solutions in cuvettes. *In vitro* calibration: Fura-2 pentapotassium salt (50 μM) was added to small volumes of buffer ((mM): K-MOPS, 10; 100; Mg^{2+} , 1; $\text{K}_2\text{H}_2\text{EGTA} + \text{Ca-EGTA}$ 10; pH 7.20) and sealed in Lucite wells. Each volume contained a different $[\text{Ca}^{2+}]$ attained and measured as described above. Ratios were determined under the digital imaging microscope from fluorescence intensities at 340 and 380 nm excitation corrected for the background fluorescence obtained in the absence of Fura-2.



evident particularly in the 340 nm image, was still present in the ratio image, indicating that it reflects localized areas of higher $[Ca^{2+}]$, rather than simple differences in dye concentration.

The precise relationship between the fluorescence ratio (340/380) and free $[Ca^{2+}]$ in the intracellular environment was determined by exposing cells to solutions of varying $[Ca^{2+}]$ in the presence of levels of Ca^{2+} -ionophore previously shown⁶ to result in equilibration of intracellular and extracellular $[Ca^{2+}]$. We found that the fluorescence ratio varied in response to changes in cellular $[Ca^{2+}]$ in a manner virtually identical to that of free dye in solution (Fig. 1b). The *in situ* calibration curve was used in turn to calculate $[Ca^{2+}]$ at each point within the cell (as described in Fig. 3). Figure 3a shows the resulting map of spatial variations of $[Ca^{2+}]$ within this cell. Intracellular variations in $[Ca^{2+}]$ are displayed as a solid surface whose height at each pixel is proportional to pCa . This image shows quite strikingly that Ca^{2+} is not uniformly distributed within the smooth muscle cell, but is present at higher concentrations inside the nucleus than in the cytoplasm. It is also present at higher concentrations in sub-sarcolemmal sites whose distribution is remarkably similar to what has been presumed to be SR in other smooth muscles⁸. Further support for the assumption that these regions reflect $[Ca^{2+}]$ inside the SR comes from observations of Fura-2 fluorescence in skinned smooth muscle cells. In saponin-skinned cells, where the SR is intact, areas of high $[Ca^{2+}]$ along the cell margins were apparent, whereas in cells skinned with Triton X-100, which permeabilizes both sarcolemma and SR, these areas were no longer detectable (G. J. Kargacin, D.A.W. and F.S.F., unpublished observations). Mean $[Ca^{2+}]$ over any region in these cellular maps was calculated using interactive graphics software to define the boundaries of the selected regions. Cytoplasmic $[Ca^{2+}]$ in 45 cells was 137 ± 13 (s.e.m.) nM, which compares favourably with other determinations made in the same cell type using other techniques (129 ± 4 nM ($n=22$), as measured from quin2 fluorescence in cell suspensions⁶ and 163 (148 with Na^+ correction) ± 20 nM ($n=16$) using a Ca -sensitive microelectrode; H. Yamaguchi, per-

sonal communication). Nuclear $[Ca^{2+}]$ in resting smooth muscle cells was significantly higher than cytoplasmic, averaging 245 ± 20 nM in 13 cells and being 220 nM in the cell shown in Figs 2 and 3a. Note that when $[Ca^{2+}]$ is calculated from measurements of total cell fluorescence (for example, as in fluorometric measurements using cells in suspension), fluorescence contributions from the nucleus and SR would result in an overestimate of cytoplasmic $[Ca^{2+}]$ by 8–10%. This source of contamination can be significantly reduced by using shorter dye loading times to reduce dye entry into the SR (see also Fig. 2 legend).

The observation that free $[Ca^{2+}]$ within the cell nucleus appears to be higher than in the cytoplasm is an unexpected finding which might be explained if intranuclear $[Ca^{2+}]$ were regulated by nuclear membrane-dependent processes, or may represent an artefact resulting from modified behaviour of the dye in the nuclear environment. This latter explanation seems unlikely in the light of several findings. One finding is that the dye inside the nucleus has the same rotational freedom (as measured by fluorescence emission anisotropy) as that in the cytoplasm, suggesting that the dye is not bound to highly immobile intracellular sites (see Fig. 1b). An even more direct test of this possibility was obtained in experiments where cells were exposed to the Ca^{2+} -selective ionophore ionomycin, which resulted in collapse of the $[Ca^{2+}]$ gradient between nucleus and cytoplasm, as well as between SR and cytoplasm (see Fig. 3b). This was also seen in ionomycin-treated cells in which cytoplasmic $[Ca^{2+}]$ was maintained below normal by incubation of cells in external $[Ca^{2+}]$ levels below 1.8 mM. The maps derived from the fluorescence ratio (340/380) revealed no differences between nucleus, SR and cytoplasm, although the higher dye content of the nucleus was still clearly evident in non-ratio images (not shown). Finally, while not all cells so far studied in this manner (human neutrophils, mouse lymphocytes, BALB/c 3T3 cells) exhibit a higher $[Ca^{2+}]$ in the nucleus than in the cytoplasm, all these cells did exhibit different intranuclear $[Ca^{2+}]$ from that of the cytoplasm, suggesting that Ca^{2+} regulation at the nuclear-cytoplasmic interface may be a general cellular property.

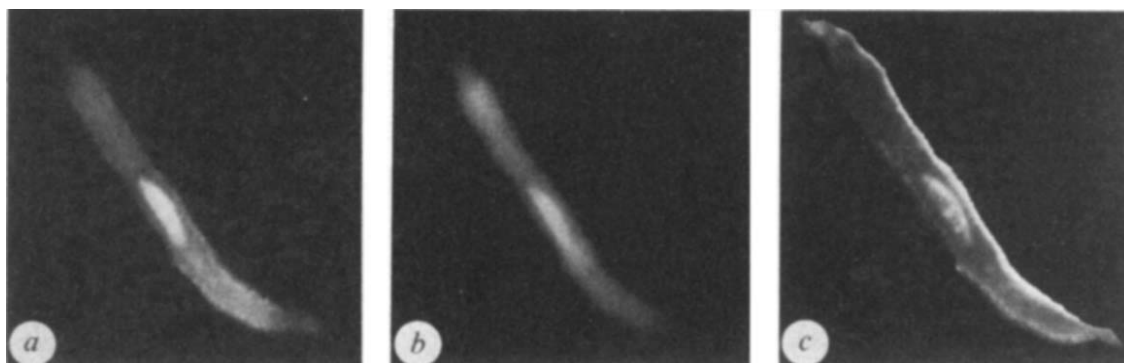


Fig. 2 Images of Fura-2 fluorescence in a single smooth muscle cell. *a*, 340 nm excitation, 500 nm emission; *b*, 380 nm excitation, 500 nm emission; *c*, ratio 340/380 image. Cell length, 150 μ m; maximum cell diameter, 11 μ m. All images were obtained as described in Fig. 1a legend. The fluorescence image displayed in *a* indicates a large degree of intracellular heterogeneity in fluorescence intensity, reflecting regional differences in dye and Ca^{2+} concentration and also that the dye can enter subsarcolemmal sites (SR). The ratio image (*c*) indicates that the cellular ionized $[Ca^{2+}]$ is apparently higher in the SR and nucleus. To determine if the high $[Ca^{2+}]$ measured over the nucleus merely reflects high $[Ca^{2+}]$ within the perinuclear space projected, because of the large depth of focus of the fluorescence microscope, onto the profile of the nucleus, we digitally calculated the image to be expected for this possibility. We assumed that intranuclear and cytoplasmic $[Ca^{2+}]$ were equal and that the perinuclear space was 0.2 μ m wide, containing a 10-fold higher $[Ca^{2+}]$. Nuclear dimensions were those determined from the cell displayed in *a-c*. The expected image was determined by convolving this model cell with the point spread function of the optical system which was determined as described previously⁷. A profile of $[Ca^{2+}]$, determined midway through the nucleus of the resultant image (results not shown), differed considerably from that observed in the actual image in that $[Ca^{2+}]$ in this image was higher along the periphery of the nucleus than over its centre. Just the opposite pattern was actually observed. A similar procedure was used to assess the contribution of SR and nuclear fluorescence to $[Ca^{2+}]$ measured in the cytoplasm. The cell was modelled as a cylinder, 150 μ m long with SR distributed in an outer annulus 1 μ m wide, encircling a cylinder of cytoplasm 10 μ m diameter. The nucleus (2 μ m wide, 10 μ m long) occupied a central position. Fluorescence intensities in each model area were calculated from the mean values in Fig. 2 at each wavelength. This model was convolved with the point spread function of the microscope. In the mid-focal plane of the model, at points adjacent to and significantly away from the nucleus, calculations revealed that the $[Ca^{2+}]$ would be overestimated by $\sim 15\%$ and 3% in these two locations, respectively, due to contribution of out-of-focus information from non-cytoplasmic sites.

Methods. Fura-2⁸ was introduced into these cells by incubating small aliquots (300 μ l) of cells in amphibian physiological saline (APS) at 30 °C with 0.3 μ M Fura-2/AM (the acetoxymethyl ester form of the dye). After 60–90 min of incubation, cellular esterases had cleaved and entrapped 20–100 μ M of the working form of the dye (as determined fluorometrically in cell suspensions), suitable concentration for monitoring most physiological processes under investigation. Substantial dye loading into the SR could be achieved by simply increasing the duration of the loading phase to ~ 180 min and is probably enhanced by the peripheral location of the SR in this cell type. The images displayed here and in Fig. 3 were all obtained after loading for 180 min (cytosolic Fura-2 concentration 150–160 μ M). Dye which had been loaded and cleaved by smooth muscle cells displayed spectral properties characteristic of free dye (acid) in solution².

Fig. 3 Maps of the spatial variation in $[Ca^{2+}]$ in smooth muscle cells. Profiles of pCa in a relaxed smooth muscle cell (*a*, from Fig. 2c) and an ionomycin-treated cell in the presence of normal (1.8 mM) extracellular calcium (*b*). Vertical heights at each pixel in these images are proportional to the pCa at that location within the cell. The areas of high intensity evident in the ratio image (Fig. 2c), the nucleus and subsarcolemmal sites, (presumably the SR), are clearly apparent in *a*. Average $[Ca^{2+}]$ in each of these cell areas are: cytoplasm, 130 nM; nucleus, 220 nM; SR, 500 nM. The $[Ca^{2+}]$ gradients between the cytoplasm, nucleus and SR are no longer evident in ionomycin-treated cells.

Methods. Fluorescence ratio images were converted to $[Ca^{2+}]$ using the equation $K_D[(R - R_{min})/(R_{max} - R)]\beta = [Ca^{2+}]$,

where R is the measured 340/380 ratio at each point in the cell, R_{max} and R_{min} are the fluorescent ratios of the same point in the cell, for a well-mixed solution of Fura-2 + Ca^{2+} and Fura-2, respectively, and β is the ratio of fluorescence at 380 nm excitation for Fura-2/Fura-2 + Ca^{2+} . The K_D of the intracellular dye was determined from the data in Fig. 1b to be 200 nM. Note that the $[Ca^{2+}]$ calculated by determining the ratio on a point-by-point basis (as above) is not equivalent to that determined by taking the ratio of the average fluorescence of large areas within the cell as the latter method would tend to underestimate the real mean $[Ca^{2+}]$ because of the non-distributive aspect of the calculation as well as the nonlinear relation between the 340/380 fluorescence ratio and $[Ca^{2+}]$. $[Ca^{2+}]$ values were converted to pCa and displayed as a solid surface on a Lexidata Model 3400 Imageview/Solidview Graphics System. These images were constructed using a standard surface modelling system which represented the cell surface as a group (160,000) of triangular polygons. Random high-frequency noise was removed by filtering (or convolving) the image with a gaussian filter whose width at half-height was 3 pixels.

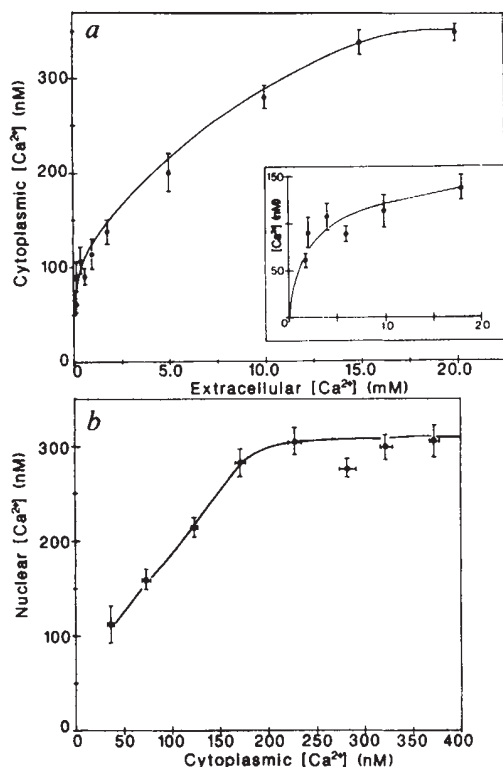
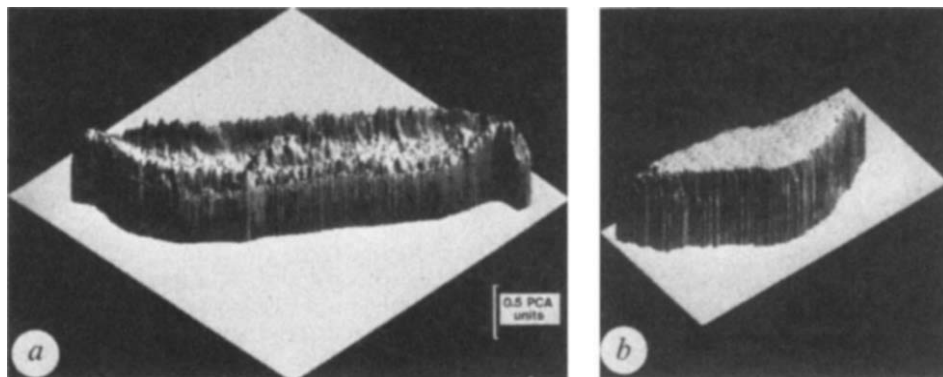


Fig. 4 Dependence of cytoplasmic and nuclear $[Ca^{2+}]$ on extracellular and cytoplasmic $[Ca^{2+}]$, respectively. *a*, Cytoplasmic $[Ca^{2+}]$ was determined in randomly selected areas of multiple smooth muscle cells as a function of external $[Ca^{2+}]$. Results are expressed as mean $\pm$ s.e.m. with a minimum of 10 observations for each point. The inset shows, on an expanded scale, the relationship between cytoplasmic $[Ca^{2+}]$ and extracellular $[Ca^{2+}]$ below 2.0 mM. As can be seen, cytoplasmic $[Ca^{2+}]$ was relatively constant in the face of a large change in the transmembrane $[Ca^{2+}]$. The relative constancy of cytoplasmic $[Ca^{2+}]$ might reflect saturation of carrier-mediated Ca^{2+} influx, or the operation of a pumping mechanism with a high Hill coefficient (>2.8). This Hill coefficient is inferred from the observed dependence of cytoplasmic $[Ca^{2+}]$ on extracellular $[Ca^{2+}]$, assuming that Ca^{2+} permeability is fixed and that influx increases 36-fold at 0.5–18 mM extracellular $[Ca^{2+}]$ which, in the steady state, must be accompanied by a similar increase in Ca^{2+} pumping out of the cell. This requires that over the observed range of cytoplasmic $[Ca^{2+}]$ (130–350 nM), the Hill coefficient for Ca^{2+} pumping is 2.86, a high level of positive cooperativity. *b*, Dependence of nuclear $[Ca^{2+}]$ on cytoplasmic $[Ca^{2+}]$. The cytoplasmic $[Ca^{2+}]$ values determined in *a* were placed into 50-nM groupings ((0–49, 50–99, ..., 350–399) irrespective of the extracellular $[Ca^{2+}]$ which produced each cytoplasmic $[Ca^{2+}]$). These values were then averaged and the respective nuclear $[Ca^{2+}]$ values plotted as a function of average $[Ca^{2+}]$ for each cytoplasmic $[Ca^{2+}]$ range. All results are presented as means $\pm$ s.e.m. Lines were fitted to the data points by eye.

The observation of a $[Ca^{2+}]$ gradient between both cytoplasm and extracellular space as well as between nucleus and cytoplasm suggests that both the plasma membrane and nuclear membrane have important Ca^{2+} -regulatory functions. To obtain further insights into the existence and nature of these processes, we exposed smooth muscle cells to solutions of varying extracellular $[Ca^{2+}]$ and determined the steady-state distribution of Ca^{2+} across the sarcolemma and nuclear membrane (Fig. 4). A strong dependence of cytoplasmic $[Ca^{2+}]$ on extracellular $[Ca^{2+}]$ was seen only below 0.5 mM (Fig. 4). By contrast, cytoplasmic $[Ca^{2+}]$ changed by no more than 3-fold as $[Ca^{2+}]_{out}$ was varied over a 36-fold range from 0.5 to 18 mM. This may be indicative of the involvement of a saturable carrier protein mediating Ca^{2+} influx in the plasmalemma or an outwardly directed Ca^{2+} pump having a large Hill coefficient (~ 3) between 130 and 350 nM $[Ca^{2+}]$ (see Fig. 4 legend). The existence of an inverse relation between Ca^{2+} 'permeability' and extracellular concentration has in fact been demonstrated previously for these cells⁵.

The changes in cytoplasmic $[Ca^{2+}]$ induced by varying extracellular $[Ca^{2+}]$ allowed us to observe the dependence of intranuclear $[Ca^{2+}]$ on cytoplasmic $[Ca^{2+}]$. As can be seen in Fig. 4b, although nuclear $[Ca^{2+}]$ closely followed changes in cytoplasmic $[Ca^{2+}]$ below the resting level (130 nM), it was virtually unaffected by increases in cytoplasmic $[Ca^{2+}]$ above this value, thereby revealing for the first time membrane-dependent mechanisms for regulating intranuclear $[Ca^{2+}]$. We doubt that higher intranuclear $[Ca^{2+}]$ is the simple result of a Donnan effect of excess negative charge in the nucleus relative to the cytoplasm. Ionomycin should not directly affect a Donnan gradient yet does abolish the observed nuclear-cytoplasmic difference. Furthermore, a Donnan effect would predict a constant nuclear to cytoplasmic free $[Ca^{2+}]$, in contradiction to the results shown in Fig. 4b, and should also influence the distribution of all permeant ions between nucleus and cytoplasm; however, studies of monovalent ion distribution by others^{9,10} indicate that any Donnan effect must be quite small. Finally, a Donnan potential that accumulates Ca^{2+} would repel Fura-2 and its Ca^{2+} complex (-5 and -3 charged, respectively), whereas the dye concentration is actually higher in the nucleus. The possibility that the nuclear membrane acts to regulate nuclear $[Ca^{2+}]$ may initially seem surprising given ultrastructural data revealing the existence of pores in this membrane¹¹, observations that dyes of relative molecular mass $<20,000$ rapidly enter the nucleus when injected into cells¹², and measurements of monovalent ion distribution between nucleus and cytoplasm revealing equivalent concentrations^{9,10}. On the other hand, there are data to support the proposal that the nuclear membranes regulate $[Ca^{2+}]$. First, the nuclear pores are not patent but filled

with electron-dense material arranged in an ordered manner¹¹. Second, transport functions are known to reside in the nuclear membranes—a system for active uptake of specific proteins has been extensively studied^{13–15} and, particularly germane to the present proposal, a Ca^{2+} -ATPase has been reported to exist in nuclear membranes^{16,17}. Finally, measurement of total Ca^{2+} with the electron microprobe, while not directly comparable to the present data on free Ca^{2+} , suggest that nuclear Ca^{2+} is somehow screened from the effect of increased cytoplasmic Ca^{2+} during contraction of rabbit portal-anterior mesenteric vein¹⁸, although screening is not apparent in the rabbit main pulmonary artery¹⁹.

The proposal that nuclear function may be subject to Ca^{2+} -linked regulation, as are many cytoplasmic processes, is certainly supported by recent observations revealing the presence of the Ca^{2+} -linked modulatory protein, calmodulin, within the nucleus of various cells²⁰, as well as *in vitro* observations revealing a Ca^{2+} or calmodulin sensitivity, for several known intranuclear

enzymes^{21–23} and for prolactin gene expression²⁴. It remains to be seen which nuclear processes are indeed regulated by Ca^{2+} *in situ* and if and when during cell activity nuclear $[\text{Ca}^{2+}]$ changes. The demonstrated ability to measure $[\text{Ca}^{2+}]$ with a high degree of spatial resolution in single living cells opens the exciting possibility of measuring changes in $[\text{Ca}^{2+}]$ in various cellular compartments (for example, cytoplasm, nucleus and SR) during changes in physiological activity to explore such questions.

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Sequences homologous to the P mobile element of *Drosophila melanogaster* are widely distributed in the subgenus *Sophophora*

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The biological properties of the P element of *Drosophila melanogaster* differ from those of other mobile elements in this genus in that its mobility is associated with dysgenic traits that might be expected to limit its distribution or spread¹. The P element is widely distributed among natural populations of *D. melanogaster*, but is absent from strains that have been maintained in the laboratory for more than thirty years². This observation prompted speculation that the P element has recently invaded and spread horizontally through *D. melanogaster*^{3,4}, a hypothesis supported by the observation that P elements are absent from the *D. melanogaster* sibling species⁵. However, sequences homologous to P elements have been found recently in *Drosophila paulistorum*, a quite distant relative⁶. This finding undermines any simple hypothesis about the origin of P elements, and demands a thorough analysis of the distribution and conservation of the P element within the subgenus *Sophophora*. We show here that the P element is present in every species group in the subgenus *Sophophora*, but that its distribution within species groups can be discontinuous. The P homologues from different species are not identical; furthermore, their genomic localization differs from that found in *D. melanogaster*.

D. melanogaster belongs to the subgenus *Sophophora*. This subgenus has 145 species distributed among four species groups: *melanogaster*, *obscura*, *saltans* and *willistoni*⁷. We examined 20 species from the subgenus, representing all 4 species groups.

The species chosen differ in their geographical range, habitat and relatedness. The divergence of the *melanogaster* group from both the *willistoni* and *obscura* species groups is estimated to have occurred about 50 million years ago⁸. Table 1 shows the phylogenetic relationships of the species used in this study. About 100 adult flies of each species were examined carefully under a dissecting microscope to confirm their identity, and DNA was prepared from them⁹, digested with *Pvu*II, separated by electrophoresis and transferred to nitrocellulose filters¹⁰. An internal 840-base-pair (bp) *Hind*III fragment from the *D. melanogaster* P element pP25.1 was purified, labelled and hybridized to the filters (see Fig. 1). The distribution of P homologues within the subgenus under low-stringency wash conditions is shown in Table 1 (see Fig. 1 legend for details). All members tested of the *willistoni* and *obscura* groups have P-element homologues, whereas some species from the *melanogaster* and *saltans* species groups lack sequences with detectable homology to the P element. Previous investigators did not detect P homologues in *D. pseudoobscura*², but this may be due to differences in the hybridization and wash conditions, or to intraspecific differences.

The filters were washed subsequently at increasing stringencies to estimate the degree of similarity between P homologues from the different species and the *D. melanogaster* P element. The representative data shown in Fig. 1 demonstrate that each species group has P-homologous sequences with distinct properties. P homologues from *melanogaster* species are weakly detectable only at low stringency (Fig. 1). More closely related P homologues are found in the *obscura* group species. The hybridization signal is detectable after low- and medium-stringency washes, but is undetectable after high-stringency washes. The most similar P homologues are found in the *willistoni* and *saltans* species groups, in which the signal is retained even when the filters are washed in 0.2 × SSC at 63 °C. Note that all *willistoni* species group members have a 0.9-kilobase (kb) *Pvu*II fragment that co-migrates with the 0.9-kb *Pvu*II fragment from the complete P element of *D. melanogaster*, whereas no member of the *saltans* group has such a fragment. This suggests that the P-homologous sequences differ between the *willistoni* and *saltans*

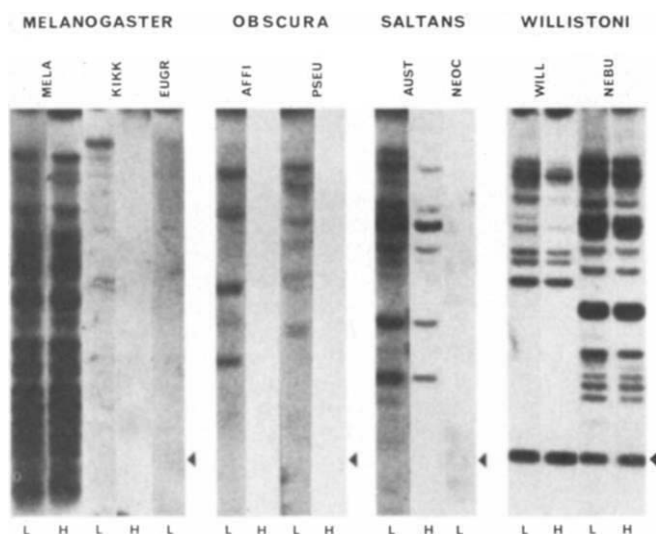


Fig. 1 Autoradiographs of Southern blots containing Sophophoran species DNA probed with P element DNA from *D. melanogaster*. DNA was prepared from adult flies using slight modifications of a published procedure⁹, digested with *Pvu*II, subjected to electrophoresis on 0.6% agarose gels and transferred to nitrocellulose¹⁰. The 840-bp internal 5' *Hind*III fragment of p π 25.1 was eluted from agarose gels, nick-translated¹⁵ to a specific activity of $0.5-1.0 \times 10^8$ d.p.m. μg^{-1} with ^{32}P -dCTP, and used as a probe. Hybridization conditions were $6 \times \text{SSC}$, 50% formamide, $5 \times$ Denhardt's solution¹⁶, 10 mM EDTA, 0.5% SDS for 12–16 h at 37 °C. The filters were washed three times for 15 min each under the following conditions: low stringency ($2 \times \text{SSC}$, 0.1% SDS; room temperature); medium stringency ($0.2 \times \text{SSC}$, 0.1% SDS; 53 °C); high stringency ($0.2 \times \text{SSC}$, 0.1% SDS; 63 °C). The stringency used is indicated below each lane, using the following abbreviations: L (low) and H (high). The species groups and species are indicated above each gel lane. Abbreviations are as follows: mela (*D. melanogaster*), kikk (*D. kikkawai*), eugr (*D. eugracilis*), affi (*D. affinis*), pseu (*D. pseudoobscura*), aust (*D. australosaltans*), neoc (*D. neocordata*), will (*D. willistoni*) and nebu (*D. nebulosa*). The arrowhead indicates the position of a 0.9-kb *Pvu*II fragment which co-migrates with the internal 0.9-kb *Pvu*II fragment of p π 25.1.

groups. The most striking observation is that the P homologues from species more closely related to *D. melanogaster* show least homology to the *D. melanogaster* P elements. Representative P homologues have been cloned from *D. willistoni* and *D. nebulosa*, and have restriction maps very similar to the map of the P element of *D. melanogaster*. A 2.9-kb P homologue from *D. nebulosa* shows extensive DNA sequence conservation compared with p π 25.1 (R.A.L. *et al.*, manuscript in preparation).

The apparent differences among P homologues prompted us to examine the location of these sequences within their respective genomes by *in situ* hybridization. In *D. melanogaster*, the P element can insert at many positions within the euchromatic portion of the genome, and although it can insert into heterochromatin, it is rarely found there^{11,12}. Figure 2 shows the results of *in situ* hybridization using either *D. melanogaster* internal P-element restriction fragments, or using P-homologous sequences cloned from *D. saltans* or *D. nebulosa*. In each of *D. nebulosa*, *D. saltans* and *D. willistoni*, the probe hybridizes extensively to centromeric heterochromatin. Only in *D. willistoni* did we regularly detect at least two euchromatic sites of hybridization. This pattern contrasts with the predominantly euchromatic location of P elements in *D. melanogaster*.

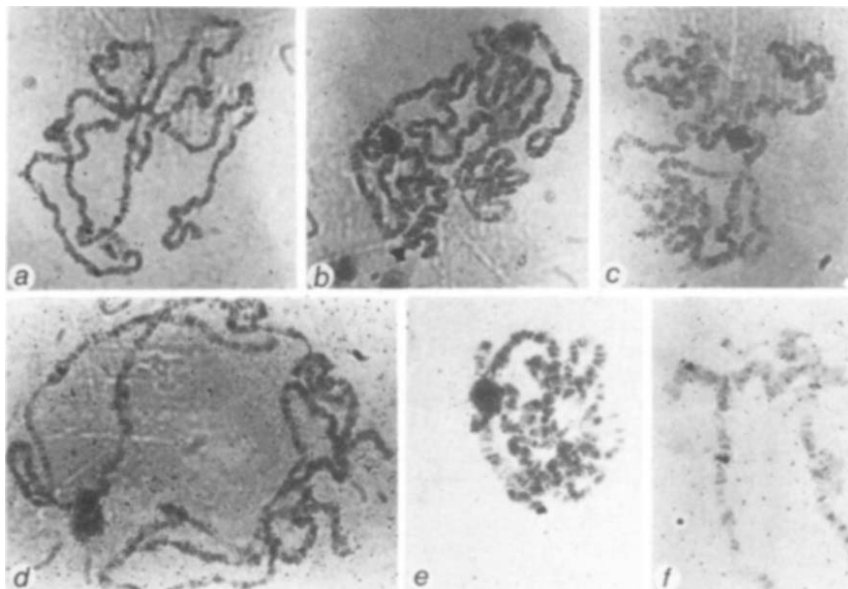
The observation that P homologues are detectable in all species groups of the Sophophoran subgenus might lead one to conclude that the P element is evolutionarily ancient, and that it appeared before the Sophophoran radiation. However, discontinuous distribution of the P homologues within species groups suggests that P elements have invaded some species after the Sophophoran radiation. If the element existed prior to the Sophophoran radiation, then the discontinuous distribution must result from the loss of the P element from some populations, but the observation that P homologues from species most closely related to *D. melanogaster* are the least conserved relative to the *D. melanogaster* P element strongly argues against a single ancient origin of the P element. The fact that P homologues are widespread but not identical in the Sophophoran subgenus suggests that Drosophilids have been subject to multiple invasions at undetermined times from an unknown external source, or alternatively, that P elements occasionally move horizontally from within the genus. The latter suggestion implies that species bearing P homologues should now have, or have had in the past, overlapping ranges. This prediction is supported by the observation that P homologues are present in species

Table 1 Phylogenetic relationships among Sophophoran species used, and distribution of P homologues

Subgenus	Species group	Subgroup	Species	P homologues
Sophophora	<i>melanogaster</i>	<i>ananassae</i>	<i>ananassae</i>	—
		<i>montium</i>	<i>kikkawai</i>	+
		<i>elegans</i>	<i>elegans</i>	—
		<i>eugracilis</i>	<i>eugracilis</i>	—
		<i>takahashi</i>	<i>takahashi</i>	—
		<i>ficuspshila</i>	<i>ficuspshila</i>	+
		<i>obscura</i>	<i>pseudoobscura</i>	+
		<i>affinis</i>	<i>affinis</i>	+
		<i>willistoni</i>	<i>willistoni</i>	+
			<i>equinoxialis</i>	+
			<i>tropicalis</i>	+
			<i>paulistorum</i>	+
			<i>paulistorum-like</i>	+
	<i>obscura</i>		<i>nebulosa</i>	+
			<i>succinea</i>	+
			<i>capricornii</i>	+
			<i>saltans</i>	+
			<i>australosaltans</i>	+
	<i>saltans</i>		<i>prosaltans</i>	+
			<i>sturtevantii</i>	+
			<i>elliptica</i>	—
			<i>neocordata</i>	—
				—

The table shows the species group, subgroup and species of each species used in this study, taken from Throckmorton⁷. All stocks were obtained from the National *Drosophila* Species Resource Center, Bowling Green State University, Ohio. The right-hand column shows whether P homologues were detected under our low-stringency wash conditions (see Fig. 1 legend).

Fig. 2 *In situ* hybridization of P elements to chromosomes of Sophophoran species. DNA was labelled to a specific activity of $3\text{--}5 \times 10^7$ d.p.m. μg^{-1} with ^{35}S -dCTP and hybridized to polytene chromosomes¹⁷. Hybridization conditions were $5 \times \text{SSPE}$, 50% formamide, $2 \times$ Denhardt's solution¹⁶, $100 \mu\text{g ml}^{-1}$ sonicated salmon sperm DNA, at 35°C for 12–16 h. Slides were washed three times for half an hour each in $2 \times \text{SSC}$ at 50°C , dried through ethanol and prepared for autoradiography¹⁸. **a**, The complete P element $\text{p}\pi 25.1$ hybridized to *D. melanogaster*, showing exclusively euchromatic localization of the P elements. **b**, P homologue from *D. nebulosa* subcloned into pUC12, hybridized to chromosomes from *D. nebulosa*, showing chromocentric localization of P elements. **c**, A gel-purified internal fragment from the 3' end of the same *D. nebulosa* homologue used in **b**, hybridized to *D. nebulosa* chromosomes to demonstrate that flanking sequences do not account for hybridization to the chromocentre. **d**, A P-homologous sequence from *D. saltans* hybridized to chromosomes of *D. saltans*. Most grains are observed over the chromocentre. Similar results were obtained when the *D. nebulosa* internal P-element probe was hybridized to chromosomes from *D. saltans* (data not shown). **e**, Hybridization of 3' internal P-element restriction fragment from *D. nebulosa* to chromosomes of *D. willistoni*. Most hybridization is to the chromocentre, although two euchromatic sites can occasionally be detected. **f**, Hybridization of the 840-bp internal 5' *Hind*III fragment from $\text{p}\pi 25.1$ used for the genomic Southern analysis hybridized to chromosomes of *D. willistoni*. Two euchromatic sites of hybridization are detected and shown here, although a third site is visible after longer exposure.



whose ranges overlap the South American and Central American range of the *willistoni* species group¹³, like the *saltans* and *obscura* groups⁷, or the nearly cosmopolitan *D. melanogaster* and *Drosophila kikkawai*⁷. The absence of P elements from some species in our sample could have several origins. First, the P element or its as yet unidentified putative vector, could have a limited host range. Second, if the source of the P element is outside the genus *Drosophila*, the source may be limited in its geographical range, perhaps constrained by its primary host. Third, the populations sampled may simply be M strains, whereas other populations of the same species may contain P homologues.

The P homologues from all three species examined by *in situ* hybridization appear to be largely confined to the heterochromatin. This heterochromatic localization may arise because heterochromatin contains a hotspot for integration of P elements in these species. If P factors are not transcriptionally active because they are confined to heterochromatin, then it would not be expected that they would spread through the population. It is interesting that in reactive strains of the IR dysgenic system of *D. melanogaster*, I elements are confined to heterochromatic regions, where presumably they are non-functional¹⁴. Molecular and biological tests of P-homologue function will be required in order to understand the origin and evolution of P elements.

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Regulation of the distribution of Ultrabithorax proteins in *Drosophila*

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The role of the bithorax complex (BX-C) in the genome of the fruitfly *Drosophila* is to specify the developmental pathways followed by cells in most of the insect's thoracic and abdominal segments¹. The BX-C has three lethal complementation groups, *Ubx*, *abd-A* and *Abd-B*, which have their major effects in progressively more posterior parts of the animal (Fig. 1)². Within the *Ubx* region³ there are four phenotypically distinct classes of mutations, *abx*, *bx*, *bxd* and *pbx*, each of which results in a part of the *Ubx* mutant phenotype^{1,4}. Here we show that these subfunction mutations all affect the distribution of *Ubx* proteins. The results support the view that the *Ubx* proteins represent the executive functions of the *Ubx* region⁵ and that the subfunction mutations affect regulatory regions required for the normal pattern of *Ubx* expression^{6,6}. In addition, we show that the region of the BX-C containing the *abd-A* and *Abd-B* complementation groups regulates the distribution of *Ubx* products.

The phenotype of *Ubx* mutations primarily affects four compartments: the posterior second thoracic compartment (T2p), the anterior third thoracic compartment (T3a), T3p and the anterior compartment of the first abdominal segment (A1a). In *Ubx* mutants, the sequence T2p T3a T3p A1a is transformed to

T1p T2a T1p T2a (refs 1, 7-10). In segments A2-A7 there is also a slight anteriorwards transformation¹.

Two of the subfunction mutations appear to affect individual compartments^{11,12}; *bx*⁻ transforms T3a→T2a and *pbx*⁻ transforms T3p→T2p. In *bx*⁻ A1a is transformed to T3a and T3p→T2p, but the latter effect may be due to *cis*-inactivation of the *pbx*⁺ subfunction^{9-11,13}. In addition, *bx*⁻ mutations have some effect on A2-A7 (ref. 1). The *abx* mutations transform both T3a→T2a and T2p→T1p (refs 4, 14). It has been suggested that the basis for these mutant effects is the compartment-specific expression of four discrete subfunctions whose activity is responsible for the identities of individual compartments^{1,2}.

How do the subfunction mutations effect these transformations? Molecular analysis of the proximal region of the BX-C has revealed two transcription units⁵. One of these, the *Ubx* unit, is known to produce several transcripts and at least three

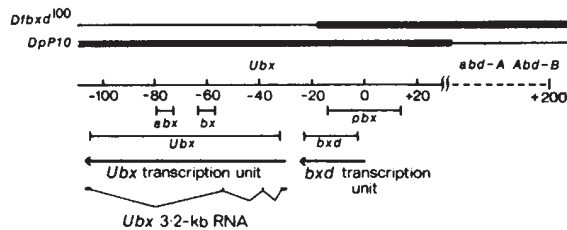


Fig. 1 The bithorax complex (BX-C)¹. There are three lethal complementation groups, *Ubx*, *abd-A* and *Abd-B*². The *Ubx* domain comprises some 120 kilobases (kb)⁵ and contains the DNA lesions associated with the *Ubx* mutations and the subfunction mutations *abx*, *bx*, *bx*^d and *pbx*¹. The ranges of the distributions of DNA lesions associated with each mutant class are indicated and are derived from ref. 3. There are two transcription units within the *Ubx* domain—the *Ubx* transcription unit and the *bx*^d transcription unit⁵. The intron-exon map of one of the processed RNAs (the 3.2-kb transcript) of the *Ubx* domain is indicated⁵. Above the DNA scale the breakpoints of two deletions are shown. The deleted regions are represented by thin lines. The breakpoint of the *Df*^{bx}¹⁰⁰ has been mapped³. Mapping of the breakpoint of *Dp*^{P10} at the DNA level has not been reported but is likely on genetic grounds to lie between *pbx* and *iab-2* in the *abd-A* region¹.

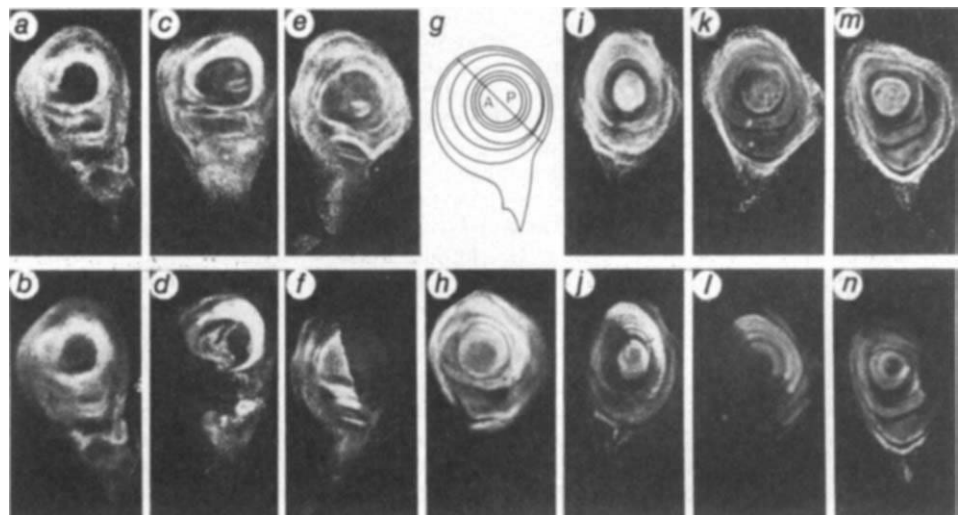
protein products^{5,15,16}. *Ubx* mutations map within this transcription unit and presumably reduce or abolish the expression of the whole set of *Ubx* proteins⁵. Different subsets of *Ubx* products may be expressed in different compartments, thus specifying different developmental pathways⁵. The subfunction mutations could interfere with these compartment-specific patterns of *Ubx* protein expression. There is evidence that *bx*⁻ mutations affect the distribution of *Ubx* proteins⁵, but the relationship between the different products of the *Ubx* unit and the subfunction mutations remains unclear, especially as two of the subfunction mutations, *pbx* and *bx*^d, map outside the *Ubx* transcriptional unit^{3,5}.

To examine the effects of the subfunction mutations on the distribution of *Ubx* proteins, we have used the monoclonal antibody FP.3.38 which recognizes an antigenic determinant encoded in the *Ubx* 5' exon¹⁶. Although we do not know whether the antibody recognizes all *Ubx* products, on protein blots of imaginal disks it recognizes at least three proteins. It does not cross-react with the products of genes outside the proximal portion of the BX-C, as *Df*^{bx}¹⁰⁰ embryos show no labelling¹⁶.

The pattern of *Ubx* protein expression in imaginal disks from wild-type third-instar larvae has been described previously^{5,16,17}. Briefly, most, if not all, of the nuclei of the third thoracic disks, the haltere and third leg disks, express *Ubx* proteins (Fig. 2). The labelling is heterogeneous and in the third leg disk there is a prominent block of labelling that is restricted to the posterior compartment of the disk. The second thoracic disks (wing and second leg disks) show weak labelling over discrete areas. In the wing disk the labelled area is posterior and includes part of the peripodial membrane and part of the disk epithelium. However, many of the nuclei in the posterior compartment of the disk are unlabelled. In the second leg disk the posterior part of the disk is labelled. The labelled area may correspond to the posterior compartment of the disk, although the signal is too low for this to be confirmed (R.A.H.W. and M.W., unpublished observations).

In *bx*³ third thoracic leg disks, *Ubx* protein expression is dramatically reduced in the anterior compartment (Fig. 2*l*). A similar effect is seen in the anterior compartment of the third thoracic dorsal disks (data not shown). In *pbx* third thoracic

Fig. 2 *Ubx* protein expression in wild-type and mutant imaginal disks. The disk whole-mounts are displayed with their anterior compartments on the left. Except for *g*, the disks in the upper line are labelled with Hoechst dye to reveal the total pattern of nuclei; the disks in the lower line are labelled with the monoclonal antibody FP.3.38, revealing the pattern of *Ubx* protein expression. *a*, *b*, Wild-type third thoracic dorsal (haltere) disks. Most, if not all, the disk nuclei express *Ubx* proteins^{5,16,17}. *c*, *d*, *abx*² homozygous third thoracic dorsal disk. The *Ubx* proteins are expressed in a complex pattern. Much of the anterior compartment shows little labelling and there are also areas of reduced labelling in the posterior compartment. The anterior-posterior (A/P) compartment boundary has not been precisely mapped in the haltere disk, but compare *d* with *f*. *e*, *f*, *pbx*¹ homozygous third thoracic dorsal disk. The *Ubx* protein expression in the posterior compartment is dramatically reduced. The sharp boundary of *Ubx* expression correlates well with the A/P compartment boundary in the wing imaginal disk²⁴. *g*, Diagram showing the position of the A/P compartment boundary in the leg disk taken from Steiner's analysis of the first leg disk²⁵. *h*, Wild-type third leg disk. The *Ubx* proteins are expressed in most, if not all, cells of the disk, but there is more labelling in the posterior compartment¹⁷. *i*, *j*, *abx*² homozygous third leg disk. There is reduced expression of *Ubx* products over the anterior compartment; however, the level of reduction is rather variable. *k*, *l*, *bx*³ homozygous third leg imaginal disk. *Ubx* protein expression is dramatically reduced in the anterior compartment. The boundary of *Ubx* expression correlates well with the position of the A/P compartment boundary (compare with *g*). *m*, *n*, *pbx* homozygous third leg disk. The *Ubx* expression is reduced in the posterior compartment, giving a pattern complementary to that in *l*.



Methods. The immunofluorescence labelling of the disk whole-mounts has been described previously¹⁶. The disks were dissected from third-instar larvae and the homozygous mutant larvae were identified by using the balancer chromosome TM6B, constructed by L. Craymer and E. B. Lewis, which carries the dominant *Tb* marker. The *bx*³ homozygous third thoracic dorsal disks are not shown as they are necrosed.

ventral and dorsal imaginal disks, a complementary pattern is observed; there is a marked reduction in the level of *Ubx* protein expression in the posterior compartments (Fig. 2f, n). In neither case is there an effect on the labelling in the second thoracic disks. These results provide a molecular demonstration of the compartment-specific effect of the *bx³* and *pbx* mutations and correlate with their compartment-specific effects on the adult cuticle^{11,12}.

The mutation *abx²* seems to resemble *bx³* in its effect on *Ubx* protein expression in imaginal disks. In the third thoracic disks the most prominent effect is a reduction of the labelling in T3a in both the dorsal and ventral disks (Fig. 2d, j). However, the phenotype is more variable than *bx³* and some disks exhibit patchy labelling in T3a and some dorsal disks also show patches of reduced labelling in T3p. This shows some correlation with the effects of *abx²* on the adult cuticle, where the most prominent effect appears to be a variable *bx*-like T3a → T2a transformation⁴. However, *abx* mutations also show a T2p → T1p transformation¹⁴ and thus the surprising aspect of the *Ubx* protein pattern in *abx²* is that this compartment seems to be unaffected.

Thus, these three subfunction mutations all affect the distribution of *Ubx* products in imaginal disks. The effects are correlated with the phenotypes of the mutants in the adult cuticle and may reasonably be supposed to be their cause. The mutations *abx*, *bx* and *pbx* therefore behave as if they affect regulatory functions required for the expression of *Ubx* products in specific areas. In the case of *bx* and *pbx*, these specific areas are T3a and T3p, respectively.

We have also examined the distribution of *Ubx* proteins in the nervous system, epidermis and mesoderm of mutant stage-13 embryos¹⁸. Here we will concentrate on the pattern of labelling in the ventral nervous system (VNS) as this is the most prominently labelled structure at this stage (Figs 3, 4). The wild-type pattern has been described previously^{5,16,17}. It exhibits a repeat unit, the *Ubx* metamer, which is out of phase with the segmental neuromere. The boundaries of the *Ubx* metameres may coincide with anterior/posterior compartment boundaries in the epidermis¹⁷ and thus *Ubx* metameres probably coincide with parasegments¹⁹. *Ubx* proteins are expressed in parasegments 5–13 in the VNS, with parasegment 6 being the most strongly labelled (Figs 4, 5).

In the embryos homozygous for the mutation *abx²*, expression of *Ubx* products is reduced in parasegment 5 (Figs 4, 5). In the wild type the anterior region of parasegment 5 is more strongly labelled than the posterior. In *abx²* some labelling remains, particularly in the posterior region. However, our interpretation of the pattern is that the whole parasegment is affected. This agrees with the analysis of *abx* mutations on the commissure pattern in the VNS where *abx* mutations were observed to affect the pattern in both posterior T2 and anterior T3 (refs 14, 20).

The effect of *abx²* shows an interesting cell-type specificity. In the wild type the midline cells at the anterior edge of parasegment 4 (that is in T1) and those in T2 are labelled¹⁷. The labelling in T1 was unexpected, as the most anterior effect of *Ubx* mutations has been reported in the adult cuticle in T2p. The labelling pattern in the midline cells might be reconciled with the pattern in the rest of the VNS if the midline cells were to migrate one parasegment length anterior. In *abx²* the *Ubx* expression in the midline cells is unaffected (Fig. 4b). This result does not support the migration hypothesis, as in *abx²* there are two clusters of midline cells labelled anterior to the main block of labelling in the VNS. Rather, it indicates that the spatial control of *Ubx* expression in the midline cells differs from that in the rest of the VNS. Another example of cell-type specificity is the observation that *abx²* seems to have no effect on *Ubx* expression in the epidermis at this stage. The tissue-specific effect of *abx* over parasegment 5 suggests that it does not act to eliminate a compartment-specific product that is required to specify the T2p developmental pathway.

In contrast to the clear effect of *abx²*, the mutations *bx³* and *pbx* have no obvious effect on the distribution of *Ubx* products in either the VNS or the epidermis in stage-13 embryos. These

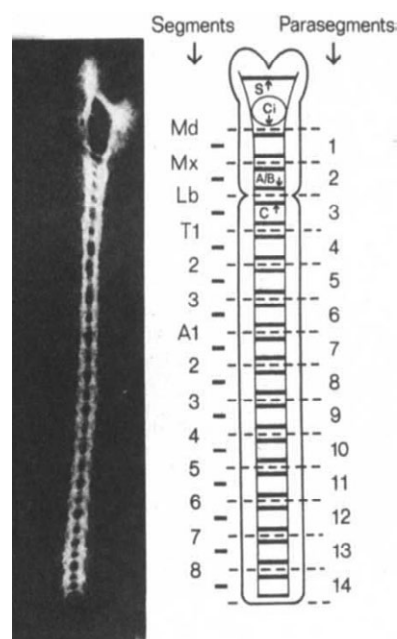


Fig. 3 Landmarks in the VNS—the commissure pattern. On the left is a dissected whole-mount of a stage-13 embryo labelled with fluorescein-coupled anti-horseradish peroxidase (HRP) antibody^{16,26}. This labelling reveals the pattern of transverse commissures (A/B and C) and lateral connectives in the VNS. On the right is an interpretation of the commissure pattern showing the segmental and parasegmental units. The head segments are represented by the supraoesophageal (S) and circular (Ci) commissures and posterior to this there is a repeat pattern of 14 parasegmental units^{17,19}. The boundaries of the parasegments are indicated on the right and probably coincide with the boundaries of the *Ubx* metameres in the VNS¹⁷. The boundaries of the *Ubx* metameres appear to lie on the anterior edge of commissure C (refs 16, 17). The segmental boundaries are indicated on the left and lie between commissure C and the succeeding A/B commissure²⁷. Md, mandibular; Mx, maxillary; Lb, labial.

mutations also have little or no effect on the commissure pattern in the VNS²⁰. We have also examined the *Ubx* pattern in the triple mutant *abx¹ bx³ pbx* and found that this shows a very similar pattern to *abx²*. (In *abx¹ bx³ pbx* the reduction in *Ubx* expression appears to be more severe than in the *abx²*. Whether this is due to the difference between the two *abx* alleles or to the influence of the *bx³* and *pbx* mutations has not been investigated.) The lack of effect of the *bx* and *pbx* mutations at this stage contrasts with their clear effects on *Ubx* expression in imaginal disks and suggests that these mutations affect functions which are specifically required late in development or in the imaginal cell lineages.

While the subfunction mutations discussed above appear to reduce *Ubx* protein levels in rather specific locations, the effect of *Tpbxd¹⁰⁰* extends over the whole range of the *Ubx* protein distribution⁵ (Figs 4, 5). Parasegments 5 and 6 are well labelled and, compared with the wild type, the labelling seems greater in the posterior part of parasegment 5 and less in parasegment 6. Parasegments 7–11 show an abnormal pattern, with fewer nuclei clearly labelled. There is a severe reduction in labelling from parasegment 11 to parasegment 12. In the wild-type a similarly severe reduction in labelling is seen between parasegment 12 and parasegment 13 (Fig. 5). This widespread effect of *bx*d mutations on *Ubx* expression correlates with their extensive phenotypic effects¹.

The second type of regulation that we have investigated is the influence on *Ubx* protein distribution of the centromere-distal region of the BX-C, containing the *abd-A* and *Abd-B* complementation groups. Deletion of this distal region in *DpP10;DfP9* homozygotes results in a transformation of parasegments 7–13 to parasegment 6 (refs 1, 10). If high-level *Ubx*

Fig. 4 *Ubx* protein expression in the VNS in wild-type and mutant stage-13 embryos. Dissected embryo whole-mounts were labelled with FP.3.38 to reveal *Ubx* proteins and with anti-HRP to reveal the commissure pattern. The parasegmental boundaries are indicated and are derived by superimposition of the anti-HRP pattern on the FP.3.38 pattern. *a*, Wild type. The *Ubx* proteins are expressed in parasegments 5–13. The most prominent expression is in parasegment 6. The labelling declines posteriorly and drops sharply in parasegment 13 where only four nuclei are well labelled at this stage. *b*, *abx*² homozygous embryo. The labelling is wild type apart from a reduction in parasegment 5. Note that the midline cells anterior to parasegments 4 and 5 are unaffected. *c*, *abx*¹, *bx*³ *pbx* homozygous embryo. Similar to *b* but slightly greater reduction of labelling in parasegment 5. *d*, *Tpbxd*¹⁰⁰. Altered pattern in parasegments 5–13. Note parasegments 5 and 6 are very similar. Compared with wild-type, fewer nuclei are clearly labelled in parasegments 7–11. Parasegment 12 shows a pattern similar to wild-type parasegment 13. *e*, Homozygous *DpP10; DfP9* embryo. This mutation leaves the *Ubx* domain intact but removes *abd-A* and *Abd-B* (see Fig. 1). Parasegment 5 is unaffected, but parasegments 6–13 exhibit the wild-type pattern of parasegment 6. *f*, *Pc*³ homozygous embryos. There is labelling over the whole VNS. Although labelling intensity declines from anterior to posterior, most parasegments exhibit a pattern similar to that seen in wild-type parasegments 7–12.

Methods. Embryos were dissected from heterozygous stocks and labelled as described previously¹⁶. The stocks and the fraction of embryos showing the mutant phenotypes illustrated above were: *abx*²/*TM1*, 8/41; *abx*¹ *bx*³ *pbx*/*TM1*, 12/36; *Tpbxd*¹⁰⁰/*TM1*, 11/42; *DpP10; DfP9*/*DfP9*, 7/31; *Pc*³/*TM1*, 5/36. The above frequencies are all consistent with the expected 1/4 homozygous mutant progeny. Of 24 embryos from *bx*³/*TM1* stocks and 29 embryos from *pbx*/*TM1* stocks, none showed any clear deviation from the wild-type pattern.

expression is a prerequisite for the parasegment 6 developmental pathway, then in *DpP10; DfP9* homozygotes parasegments 7–13 should express high levels of *Ubx* protein, and this is indeed what we find (Figs 4, 5). Thus it appears that in the wild type, *abd-A* and/or *Abd-B* regulate *Ubx* protein distribution in parasegments 7–13. Such interactions between homoeotic genes have been proposed²⁸ and the regulation of the distribution of *Antennapedia* transcripts by genes in the BX-C has provided a clear example²¹. This interaction is investigated and discussed more fully elsewhere²².

We would like to note here how this interaction contributes to our understanding of other mutant effects. The first is tentative and concerns the *Tpbxd*¹⁰⁰ phenotype. In embryos homozygous for *Tpbxd*¹⁰⁰, the effect on *Ubx* product distribution shares two features with the *DpP10; DfP9* effect in that many segments are affected and the pattern is altered in both VNS and epidermis. In addition, the sharp decrease in *Ubx* protein level in the wild-type pattern in parasegment 13 occurs in parasegment 12 in the *Tpbxd*¹⁰⁰ pattern. The decrease in wild-type parasegment 13 is part of the regulation of *Ubx* product distribution by genes in the centromere-distal region of the BX-C, as it is not observed in *DpP10; DfP9* embryos (see also ref. 22). These features suggest that an important part of the effect of *Tpbxd*¹⁰⁰ on *Ubx* protein distribution may be its effect on the interactions between homoeotic genes.

The second case is the effect of *Polycomb* (*Pc*) on *Ubx* protein distribution. The labelling pattern in the VNS of homozygous *Pc*³ embryos is shown in Figs 4 and 5 and is similar to that reported previously⁵. The whole of the VNS is labelled, and although the intensity of labelling is quite variable, it generally tends to be higher anteriorly and to tail off posteriorly. The *Pc* gene has been postulated to act as a repressor of BX-C genes¹ and thus in *Pc* mutant embryos *Ubx* should be derepressed. However, *Ubx* protein products are not expressed at the high level found in parasegment 6 in the wild type. This is readily explained if the other genes of the BX-C are also expressed along the length of the animal in *Pc* embryos. The regulation of *Ubx* protein expression by *abd-A* and/or *Abd-B* (perhaps together with other homoeotic genes) then results in the observed ubiquitous moderate level of *Ubx* protein expression.

Our results support the model that the *Ubx* proteins represent the executive functions of the *Ubx* complementation group³, as the subfunction mutations all affect the distribution of *Ubx* products. The subfunction mutations appear to map outside

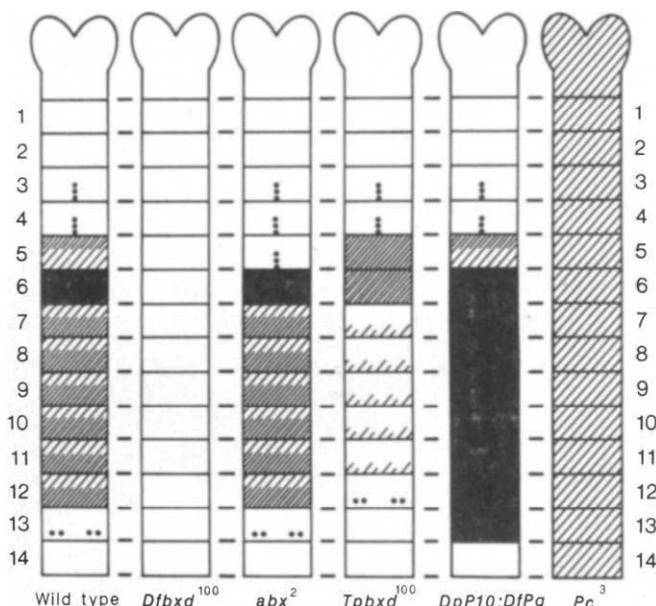
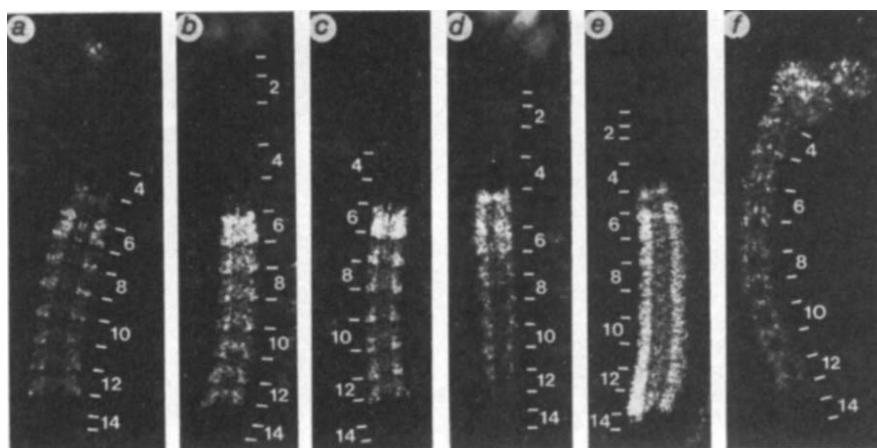


Fig. 5 Schematic diagram of *Ubx* protein expression in the VNS in wild-type and mutant embryos. The parasegmental units are indicated at the sides of the figure (see Fig. 4 for descriptions). *DfP10; DfP9* homozygous embryos lack the *Ubx* domain but contain the *abd-A* and *Abd-B* regions (see Fig. 1). They exhibit no labelling with FP.3.38 (ref. 16).

known coding sequences for *Ubx* products^{3,5} and behave as mutations in *cis*-regulatory sequences¹¹. However, it is not clear to what extent the specificity of their effects reflects the existence of specific wild-type regulatory functions rather than being a property of the mutations themselves. It has already been noted that the subfunction mutations differ in the nature of their DNA lesions as well as in their location³. For example, most *bx* mutations that have been examined are insertions of the *Gypsy* transposable element³ and developmentally regulated activity of the *Gypsy* promoter elements²³ may be important for their specific phenotypes.

Investigation of the patterns of *Ubx* transcript¹⁵ and protein¹⁷ distribution in wild-type embryos and larvae suggests a complex

programme of spatial and temporal control. Although we know little about the regulation of these patterns, we have established here that, in the stage-13 embryo, interactions between the genes of the BX-C have an important role. The key to understanding the subfunction effects may well lie in the interactions regulating the pattern of *Ubx* expression rather than in the individual *Ubx* products.

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Contrabithorax mutations cause inappropriate expression of Ultrabithorax products in *Drosophila*

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The mutation *Contrabithorax* (*Cbx*) has played an important part in the recognition of the role that genes of the bithorax complex (BX-C) play in the control of development. *Contrabithorax*, a dominant mutation which maps in the proximal or Ultrabithorax domain of the BX-C, results in the transformation of structures of the second thoracic segment (T2) into homologous structures of T3 (ref. 1). Recessive mutations and deletions of the same chromosome region have the reverse effect, transforming T3 into T2. This reciprocal relationship between the effects of dominant (gain of function) and recessive (loss of function) mutations suggested that products of the BX-C were both necessary and sufficient to specify the unique developmental pathways of different segments². Here we report that the original *Contrabithorax* mutation (*Cbx*¹) and two other dominant mutations in the Ultrabithorax domain, *Cbx*³ (ref. 3) and *Haltere mimic* (*Hm*)^{3,4}, all cause the inappropriate expression in T2 of products from the *Ultrabithorax* (*Ubx*) transcription unit. This strongly supports the suggestion that these products execute the functions of the proximal or Ultrabithorax domain of the BX-C⁵.

The DNA lesion in *Cbx*¹ is an insertion of ~17 kilobases (kb) into intron sequences of the *Ubx* transcription unit. The inserted sequences duplicate, in inverse orientation, a region normally located 27-44 kb upstream from the *Ubx* transcription start site^{5,6}. Two models have been proposed to explain the effects of this lesion on the development of cells in T2 (ref. 6). The first model suggests that the inserted DNA encodes a function that is required for the development of T3. The new location of this DNA fragment in the *Cbx*¹ mutation may then induce the inappropriate expression of this function in cells of T2, causing them to follow the T3 developmental pathway. The second model suggests that the *Cbx*¹ insertion interferes with the normal spatial regulation of the *Ubx* transcription unit itself, and that it is the inappropriate expression of *Ubx* products in T2 which changes the developmental pathway of these cells.

We have approached this question by examining the distribution of *Ubx* transcripts and proteins in the wing imaginal disks of larvae homozygous for the *Cbx*¹ mutation. In the wild type, there is little expression of *Ubx* in the wing disks (which will give rise to most of the dorsal structures of T2 in the adult), but *Ubx* transcripts and proteins are relatively abundant in the homologous disks of T3, which will give rise to the haltere, and in the T3 leg disks^{7,8}. In *Cbx*¹ homozygotes, *Ubx* transcripts and proteins are expressed in regions of the wing disk at levels comparable with those found in the normal T3 disks (Figs 1, 2).

The patterns of *Ubx* expression can best be seen in whole-mounts of wing disks which have been labelled for *Ubx* proteins (Fig. 2). Although details of the protein distribution vary from disk to disk, a high level of *Ubx* proteins is usually observed throughout most of the posterior compartment of *Cbx*¹ wing disks, with patches of expression also in the anterior compartment. This distribution correlates well with the cuticular phenotype seen in adults homozygous for the *Cbx*¹ mutation⁹. The posterior compartment of the second thoracic appendage is frequently transformed to T3, whereas the anterior compartment is transformed less frequently, and only in patches. This correspondence between cuticular phenotype and the expression of *Ubx* strongly suggests that the inappropriate expression of *Ubx* products is the cause of the observed transformations.

We have also examined the distribution of *Ubx* products in wing disks carrying the *Cbx*³ and *Hm* mutations. Both of these mutations have phenotypes analogous to that of *Cbx*¹, but the regions of T2 which are transformed to T3 are different for each of the three mutations³. *Cbx*³ results in a variable transformation which at its most extreme affects the entire anterior compartment of the wing and notum, but does not transform the posterior compartment (Fig. 3). *Ubx* proteins are also expressed in variable, but often extensive, regions in the anterior compartment of the *Cbx*³ wing disks, and the boundary of *Ubx* expression frequently defines a line at or close to the location of the anterior/posterior compartment boundary. The *Hm* mutation has a much more uniform phenotype: the entire wing blade is transformed to haltere tissue, but the notum is unaffected. In this case, a relatively constant pattern of *Ubx* expression is observed in the wing disk. Regions of the disk which will form the wing blade express high levels of *Ubx* proteins, but specific cells in other regions of the disk also express *Ubx* (Fig. 2).

None of these is simply a 'constitutive' mutation, resulting in the indiscriminate expression of the *Ubx* gene. In the case of *Cbx*¹ the only structures of the third-instar larva in which we have seen an effect of the mutation are the T2 dorsal and ventral disks. In other disks (for example, eye-antennal), and in other tissues, which do not normally express *Ubx* (for example, brain lobes, midgut), neither *Ubx* transcripts nor proteins are detectable (Fig. 1 and data not shown). Abnormally high levels of *Ubx* products are observed, however, in parasegment 5 of the embryonic nervous system (data not shown). In the case of *Hm* we have seen the inappropriate expression of *Ubx* in certain epidermal cells other than the wing disks, including cells of the anterior spiracle, but not in endoderm or most other tissues.

It is not yet clear how the effects of these dominant 'gain of function' mutations relate to the normal spatial regulation of

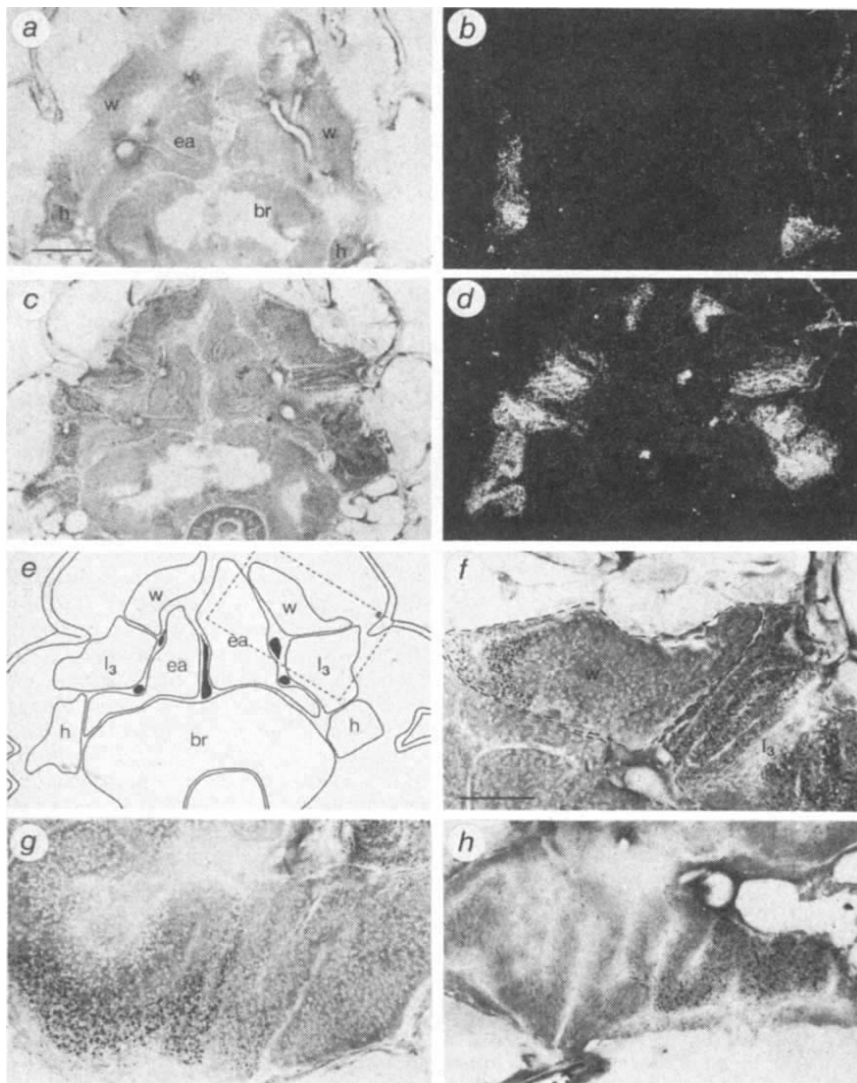
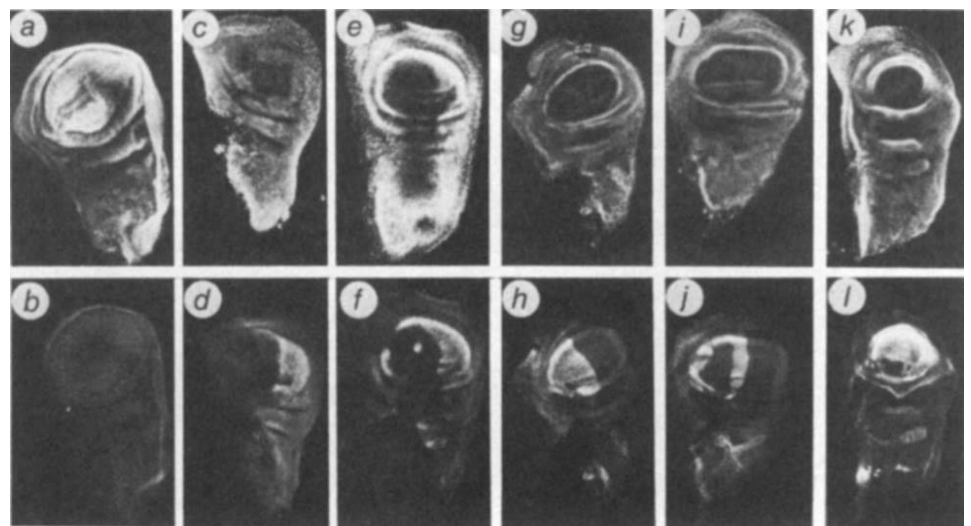


Fig. 1 Hybridization of *Ubx* probes to *Cbx* and *Hm* tissue sections. *a, b*, Bright- and dark-field photograph, respectively, of an autoradiograph of a horizontal section through the anterior of a wild-type third-instar larva. The *Ubx* probe hybridizes to the haltere disks (*h*) of T3, but not to the wing disks (*w*) of T2, nor to the eye-antennal (*ea*) disks or brain (*br*). (See ref. 7 for further details of the wild-type pattern.) *c, d*, Bright- and dark-field views of a comparable autoradiograph from a *Cbx*¹/*Cbx*¹ larva. The outlines of structures visible in this section are indicated in *e* and the boxed region is shown enlarged in *f*. The *Ubx* probe hybridizes to the disks of T3 (haltere and third leg (*l*₃)), and also to regions of the wing disk, but not to eye-antennal disks or brain. *g*, Wing disk from *Cbx*¹/*Cbx*¹ third-instar larva, showing extensive transcription of *Ubx*. *h*, Wing disk from a *Hm*/+ third-instar larva, again showing a large region of *Ubx* transcription. Scale bars: *a-d*, 100 μ m; *f-h*, 50 μ m.

Methods. Probe preparation and hybridization conditions were as described in Fig. 3 legend of ref. 7. Note that the hybridization probe used is single-stranded (synthesized on the M13 template A113), and will therefore hybridize only to 5'-3' transcripts from the 5' exon of the *Ubx* transcription unit, and not to any transcripts originating from a promoter in the DNA of the *Cbx*¹ insertion, which would traverse the *Ubx* 5' exon in reverse orientation. Autoradiographic exposures were as follows: *a, b*, 15 days; *c-h*, 24 days.

Fig. 2 *Ubx* protein expression in wild-type and mutant wing imaginal disks. In the upper line the disks are labelled with the DNA-binding dye Hoechst 33258 to reveal the total pattern of nuclei. In the lower line the disks are labelled with the monoclonal antibody FP.3.38 to reveal the pattern of *Ubx* protein expression (see ref. 8 for details of this procedure). The disks are oriented with the anterior compartment on the left. *a, b*, Wild type. There is weak labelling on the posterior side of the disk, principally in the widely spaced nuclei of the peripodial membrane. *c-f*, *Cbx*¹/*Cbx*¹ disks. In *d* the labelling appears to fill the posterior compartment of the disk and the label boundary correlates well with the position of the anterior/posterior compartment boundary¹³. A more complex pattern is observed in the disk shown in *e* and *f*. It is not clear whether the entire posterior compartment is labelled, and there is patchy labelling in the anterior compartment. *g-j*, *Cbx*³/+ disks. In *h* the labelling appears to be restricted to the anterior compartment and, within this, principally in the presumptive distal region of the disk. The sharp boundary in the distal region probably coincides with the anterior/posterior compartment boundary. The disk in *j* shows an example of less extensive expression. The sharp boundaries of *Ubx* protein expression in *h* and *j* coincide with morphological discontinuities visible in the Hoechst-labelled disks (but barely discernible in these photographs, *g* and *i*). Thus, in *g* there is a single discontinuity corresponding to the single boundary of *Ubx* expression in *h*, and in *i* there are two discontinuities in the central distal area corresponding to the boundaries in *j*. Presumably, these discontinuities reflect cell-surface differences under *Ubx* control. *k, l*, *Hm*/+ disks: *Ubx* proteins are expressed in a complex pattern, mostly over the distal region of the disk. Other *Hm*/+ disks examined showed a very similar pattern of *Ubx* protein expression.



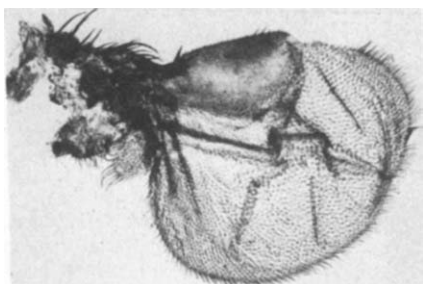


Fig. 3 The wing of a *Cbx*^{3/+} fly. Anterior is at the top and the proximal regions are to the left. The transformation of anterior structures is virtually complete in the distal regions, where the cuticle is covered with small, densely packed trichomes characteristic of the haltere. Proximally, the transformation is less complete, with many wing hinge structures remaining.

the *Ubx* gene. In the case of *Cbx*¹, there is reason to believe that the effect of the mutation on *Ubx* expression does reflect, in an aberrant way, the normal functions of the transposed DNA sequences. The *Cbx*¹ insertion arose together with a reciprocal deletion of *Ubx* upstream sequences, and was only subsequently separated by recombination¹⁰. Deletion of these sequences generates a *postbithorax* mutation¹⁰, which greatly reduces *Ubx* expression in the posterior part of the haltere disk¹¹. Thus, the deletion and relocation of these *Ubx* upstream sequences have virtually reciprocal effects on *Ubx* expression, suggesting that the transposed element normally has a role in the regulation of *Ubx*, principally in posterior compartments.

The *Cbx*³ mutation probably affects a different regulatory element. The only DNA lesion detected in this chromosome is an inversion break at the extreme 3' end of the *Ubx* transcription unit, 60 kb from the site of the *Cbx*¹ insertion⁶. This mutation allows the expression of *Ubx* in a region where it is normally never active, but has little or no effect on the expression of *Ubx* in those regions where it is normally active; flies homozygous for the *Cbx*³ mutation survive, and are virtually normal in T3 and A1 (M.E.A., unpublished observations). A second mutation with a very similar phenotype (*Cbx*^{tw1}) proves to be an inversion with one break at virtually the same site in the BX-C⁶; this suggests that both mutations affect a regulatory element located near the 3' end of *Ubx* which is involved in the repression of *Ubx* in the region immediately anterior to its normal domain of expression.

The *Hm* mutation is associated with a chromosome rearrangement broken within 4 kb of the *Ubx* transcription start site¹², suggesting that it may result from the fusion of unrelated upstream elements to the *Ubx* gene.

These three dominant mutations, of very different structure, are alike only in that they result in the expression of *Ubx* proteins in the wing disk. It seems likely that any DNA rearrangement which has this effect, however fortuitously, will result in a 'Contrabithorax' phenotype.

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Distribution of *Ultrabithorax* proteins in mutants of *Drosophila bithorax* complex and its transregulatory genes

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The phenotypic effects of mutations have classically been described in terms of their penetrance, expressivity and specificity¹. These three parameters can give insights into the wild-type function of the genes affected by the mutations; for example, the topographic specificity of homoeotic mutations provides a crucial clue to our understanding of the normal function of homoeotic genes in development. The null alleles of such genes have constant specificity², and the expectation is that these genes are expressed in all the cells of the compartments affected by their mutations. However, hypomorphic mutations of the 'selector' genes that specify compartment identity are also known which have only partial, and sometimes variable, specificity. We have now studied the distribution of protein products of the *Drosophila Ultrabithorax* (*Ubx*) gene in various mutants, both of the *Ubx* domain of the bithorax complex and of *trans-regulatory* genes predicted to affect *Ubx* expression³. We find that the topographical specificity of the mutations correlates with the distribution of *Ubx* protein. In particular, in partially transformed compartments we find sharp within-compartment variation in the level of *Ubx* protein expression. This may correspond to alternative steady states of gene activity established by cell-cell interactions, which we suggest may reflect processes of normal development involved in embryonic compartmentalization.

The *Ubx* products were visualized by immunofluorescence using a monoclonal antibody specific for the protein products of the common 5' exon of the *Ubx* transcription unit⁴. The distribution of the *Ubx* proteins was studied in imaginal disks of mature larvae. Positive label only indicates the presence of at least one of the *Ubx* products and negative label indicates the absence of all or the most abundant proteins⁴. Fate maps of the imaginal disks considered here, the wing and haltere disks^{5,6}, allow us to correlate the adult transformation with the distribution of *Ubx* products in the mutant imaginal disks (Fig. 1a).

Previous studies using this antibody have shown that the *Ubx* proteins have a non-homogeneous distribution in the wild-type imaginal disks⁷, something not expected on the basis of mutational analyses. Thus, *Ubx* proteins appear in both compartments of the haltere disk, but show a more extreme label in the posterior one. Even within compartments, certain regions regularly show more label than others (Fig. 1c). *Ubx* label also appears in the pleural region of the posterior compartment of the wing disk⁴ (Fig. 1b). This is expected because *Ubx* mosaics show transformations in the posterior mesopleura^{8,9}.

The *Ubx* domain can be subdivided into two regions. One codes for RNAs that become translated into different proteins, the *Ubx* transcription unit, while the second is a *cis-regulatory* region coding for several RNAs, possibly involved in the differential splicing of the *Ubx* transcripts¹⁰; it is in this region that *postbithorax* (*pbx*) and *bithoraxoid* (*bxd*) mutations map¹¹.

We have analysed the distribution of *Ubx* products in several mutants of the *Ubx* region. One of them, *bx*^{34c}, shows, with variable expressivity but constant specificity, partial transformations to anterior wing and mesonotal structures in the dorsal metathorax⁸. This transformation is associated with an enlarged haltere in the presumptive wing territory (compare Fig. 1c with 1d). Figure 1d shows an example of the *Ubx* products in the transformed *bx*^{34c} haltere disks. The pattern of immunofluorescence varies slightly between imaginal disks of the same genotype. It holds for all cases observed, however, that distinct patches with only weak (and in some cases absent) label

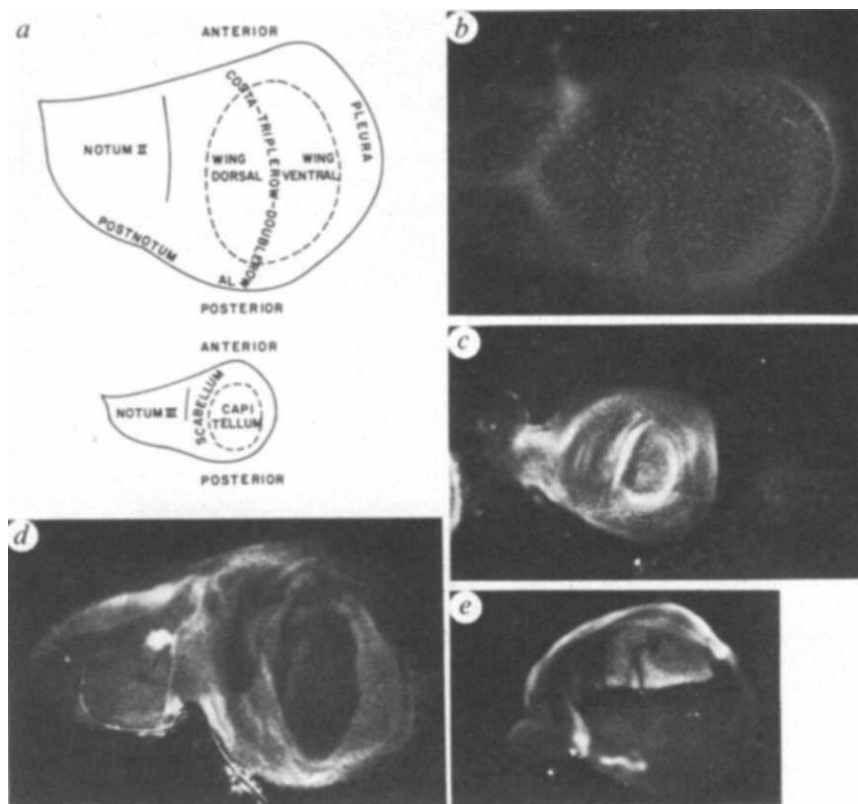
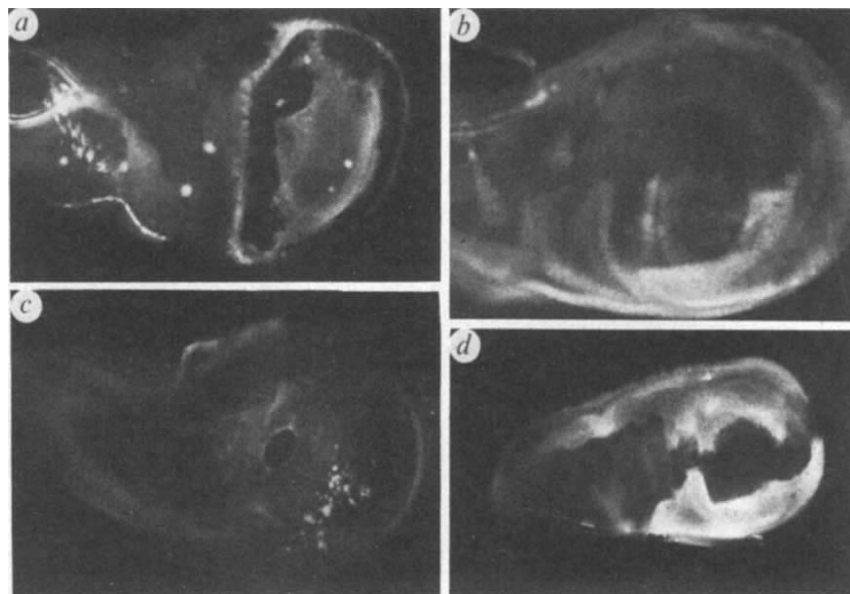


Fig. 1 Immunofluorescence patterns of *Ubx* expression. *a*, Fate maps of wing and haltere imaginal structures^{5,6}. Upper is anterior and lower posterior throughout. *b*, Wild-type wing disk; ventral view showing the posterior peripodial membrane (pleura) positively stained. *c*, Wild-type haltere disk. *d*, *bx*^{34c}/*Df*(3R)*Ubx*¹⁰⁹-transformed haltere disk with dark patches of unlabelled tissue in the anterior compartment. *e*, *pbx*¹/*Df*(3R)*Ubx*¹⁰⁹-transformed haltere disk with a large fraction of the posterior compartment showing negative staining. Note the positive label of the peripodial membrane.

Methods. Larvae were obtained from outcrosses of stocks balanced with *TM6B*, carrying a dominant marker (*Tubby*) to distinguish the genotypes to be studied. Third-instar larvae were dissected in TBS (50 mM Tris pH 7.4, 150 mM NaCl), disks mounted in polylysine-coated slides and further treated as described elsewhere⁴. The first antibody was FP3.38 ascites fluid used in a 1:200 dilution. The second antibody was goat anti-mouse IgG conjugated to fluorescein (Sigma) used at 1:50 dilution. Slides were mounted in glycerol-propylgallate-TBS and photographed with Ektachrome 400 ASA film in a Nikon microscope using filter set IF460-485. All major disks and salivary glands were studied from each mutant. Several individuals (4-10) in each case were dissected to ascertain the constance of the pattern. Controls of wild-type and *Df*(3R)*Ubx*¹⁰⁹/+ disks were run in parallel. The latter genotype consistently showed a slightly reduced intensity of staining.

Fig. 2 Immunofluorescence patterns of *Ubx* expression. *a*, *T*(2;3)*Hm*/+ transformed wing disk. The positive label in the wing blade corresponds with the adult transformation to capitellum, the spots of label observed on the presumptive notum are due to *Ubx*-positive cells of the trachea. *b*, *Cbx*¹/+ transformed wing disk. The positive label appears in discrete patches in the posterior wing region. *c*, *Pc*³*Dp*(3;3)*P5*/+ wing disk with spots of positive label in the posterior region *Dp*(3;3)*P5* is a tandem duplication of the entire bithorax complex (BX-C). Extra copies of the BX-C enhance the *Cbx*-like phenotype produced by *Pc* mutations, but never show mutant phenotype in *Pc*⁺ flies. The black hole in the middle of the disk is due to an air bubble. *d*, *Df*(3R)*red*^{P52}/*Rg-bx*^{trxA} (*Rg-bx*^{trxA}) (M. P. Capdevila, unpublished) haltere disk with patches of negative staining in the anterior region.



(Fig. 1*d*) appear in regions of the anterior compartment, corresponding in position and extent to the wing-transformed territories of the adult dorsal metathorax. The boundaries between strong and weak (or absent) signal are very precise, although along them some cells with intermediate levels of label can also be seen. Cell lineage analysis of *bx*^{34c} mutant halteres shows that even small clones, generated late in development, can include both wing bristles and trichomes with haltere characteristics. The boundaries between these two types of tissue result from late developmental decisions in cells unrelated by lineage, that is, the transformation is not clonal (G. Morata and P. A. Lawrence personal communication, and unpublished observations of this laboratory).

The *pbx* mutants show, with variable expressivity and constant specificity, transformation of posterior haltere to posterior wing structures⁸. The *pbx*¹ haltere disks (Fig. 1*e*) show sharp bound-

daries between the putative transformed (enlarged) and non-transformed tissue. The distinct patches, which here are devoid of label, correspond in position and extent to the adult wing transformations (see also ref. 12). The absence of label observed in *pbx*¹ haltere disks supports the hypothesis that the *pbx-bxd* region regulates *Ubx* in *cis* (refs 10, 13). We do not know whether the *pbx* transformations are of clonal origin.

Dominant mutations due to gain of function of the *Ubx* gene are generically designated *Contrabithorax* (*Cbx*). They are associated with molecular alterations in the DNA, within (*Cbx*¹) or near (*Hm*: *Haltere mimic*) the *Ubx* coding region (ref. 11 and W. Bender, personal communication). All the known *Cbx* mutants differ in specificity. We present here data for *Cbx*¹ and *Hm* (ref. 14 and Fig. 2). *Cbx*¹ heterozygotes show, with variable expressivity and constant specificity, a transformation of mainly the posterior wing into posterior haltere tissue, with smooth

boundaries. This transformation is not clonal¹⁵. *Hm*⁺, on the other hand, presents distal (both anterior and posterior) compartments of the wing transformed into haltere tissue (capitellum). The histotypical borders are rather sharp, but the transformation is not clonal, at least from early stages of development (M. P. Capdevila, personal communication). The mutant wing disks of both *Cbx*¹ and *Hm* retain positive label in pleural regions but also have *Ubx* products in the regions that correspond to the transformations observed in the adult (Fig. 2a, b). In both cases, sharp boundaries can be observed between labelled and unlabelled cells. A pattern of label similar to that of wild type has been detected in the mesothoracic leg disk (labelled) and in the more anterior thoracic or cephalic eye-antennal disk (no label). The heterozygotes for these mutations show a normal pattern of *Ubx* products in both haltere and metathoracic legs.

The non-clonality of *bx* and *Cbx* transformations shows that they do not result from early segregation of lineages of cells. The adult mosaic transformations probably result from interactions between neighbouring cells during the development of the imaginal disks. The discontinuity of *Ubx* label observed in alleles causing either loss or gain of function corresponds to strong quantitative differences in *Ubx* products. These boundaries probably reflect discontinuity of territories with cells in either of two steady states—one typical of full expression of the *Ubx* gene, the other of lack of *Ubx* products. This reinforcement/extinction pattern of expression in neighbouring, genetically identical cells further suggests that regulatory mechanisms are involved in the maintenance of gene activity. Therefore, we studied the pattern of expression of *Ubx* products in flies mutant for two *trans*-regulatory genes of *Ubx*; these were *Polycomb* (*Pc*)^{16,17} and *Regulator of bithorax* (*Rg-bx*)¹⁸⁻²⁰.

Mutations in *Pc* cause a *Cbx*-like transformation in the wing of heterozygotes and mutations in *Rg-bx* cause *bx*-like transformations in the halteres of heterozygotes. The *Cbx*-like phenotype produced by *Pc* is of variable expressivity but of constant specificity; haltere tissue appears in the posterior wing border and gradually goes into wing tissue. This transformation is not clonal¹⁷. The *Ubx* label of *Pc*³ wing disks appears as multiple scattered spots in the presumptive region of the adult transformations, against a wild-type unlabelled background (Fig. 2c). The transformations caused by *Rg-bx* mutations are of variable penetrance, full expressivity but erratic specificity¹⁸. These transformations are not clonal, at least from early stages of development²⁰. The haltere disks of *Rg-bx* mutants show erratic patches of unlabelled tissue, separated by sharp boundaries from fully labelled territories (Fig. 2d). The patches appear restricted to the anterior compartments of haltere disks as are the transformations seen in the adult cuticle.

Genetic arguments suggest that both *Pc* and *Rg-bx* genes are involved in the regulation of *Ubx* expression both in embryogenesis and during imaginal cell proliferation^{3,19,21}. The genetic model suggests that *Pc*⁺ acts as a repressor and that *Rg-bx*⁺ has a positive role in the expression of *Ubx* (ref. 21 and J.B. and A.G.-B., unpublished data). The present results support these arguments and indicate further that these genes could be involved in the reinforcement/extinction process suggested above. Since both *Pc* and *Rg-bx* transformations are not clonal, the reinforcement/extinction process must extend to neighbouring cells. We do not know the polarity of these influences (expressing as opposed to non-expressing cells), neither do we know which are the genes of the syntagma involved in these interactions. Possibly these mechanisms also affect selector gene expression in regeneration and *trans*-determination²².

Our results indicate that cell interactions must be involved in determining the spatial patterns of expression during imaginal disk cell proliferation. In addition, we postulate that the same mechanisms also take place in early specification. *Ubx* transcripts appear in blastoderm stages, first as uniformly distributed over the presumptive mesothorax, metathorax and first abdominal segments, and later becoming restricted to posterior mesothorax, metathorax and anterior first abdominal²³. The

extinction in the mesothorax is not complete, but remains in the posterior pleural cells. We postulate that *Cbx* mutations affect this process of spatial restrictions, retaining in part the early embryonic pattern of transcription. Why this pattern is retained in an allele-specific manner in the different *Cbx*-like mutations is not known. The reinforcement/extinction process in mutants with partial loss of function is also reminiscent of the mechanisms involved in compartment specification. Early diffused patterns of transcription over several compartments become sharply delineated in the cells of wild-type individuals, as imaginal cells mutant for hypomorphic alleles do within compartments during proliferation. Again, we do not know why these patterns are allele-specific topographically. Indeed, pattern specificity, in both normal and mutant organisms, remains the most elusive problem in developmental genetics.

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Identification of the gene responsible for human T-cell leukaemia virus transcriptional regulation

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Human T-cell leukaemia viruses (HTLVs) have genomic organization distinct from that of other replication-competent retroviruses, possessing four genes, *gag*, *pol*, *env* and χ . The unique fourth gene, χ (also referred to as *lor*), is located between *env* and the 3' long terminal repeat (LTR), encoding a protein of relative molecular mass 40,000 for HTLV-I and 37,000 for HTLV-II^{1,2}, located in the nucleus of infected cells^{3,4}. HTLV-I is the causative agent of adult T-cell leukaemia (ATL), a T-lymphocyte malignancy^{5,6}, while HTLV-II has been found associated with a T-cell variant of hairy cell leukaemia^{7,8}. Both viruses immortalize T cells *in vitro*⁹⁻¹¹. However, the mechanism of cellular transformation induced by HTLV is not known as there seems to be no common site of provirus integration in primary ATL cells¹² and the virus contains no classical oncogene sequences^{13,14}. These observations have provoked speculation that the unique and strongly conserved χ protein (85% amino-acid homology between HTLV-I and -II) is involved in HTLV leukaemogenesis. Recent mutagenesis experiments in our laboratory have shown that the χ gene is essential

for HTLV replication¹⁵. It has also been shown that the LTRs of HTLV and the related bovine leukaemia virus (BLV) are activated *trans* in virus-infected cells¹⁶⁻¹⁹, and, although such experiments did not directly demonstrate a role for the χ protein in transcriptional activation, it has been suggested that the χ protein is responsible for the transcriptional activation of the LTR and may be involved in cellular transformation^{3,4,16}. We have now developed a transient co-transfection assay which demonstrates that transcriptional activation of the HTLV LTR is mediated solely by the χ protein and that no other virus genes are required.

Others who have examined transcriptional regulation of the LTRs of HTLV or BLV have used constructions where transcrip-

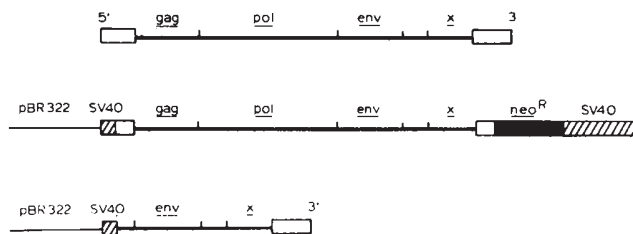
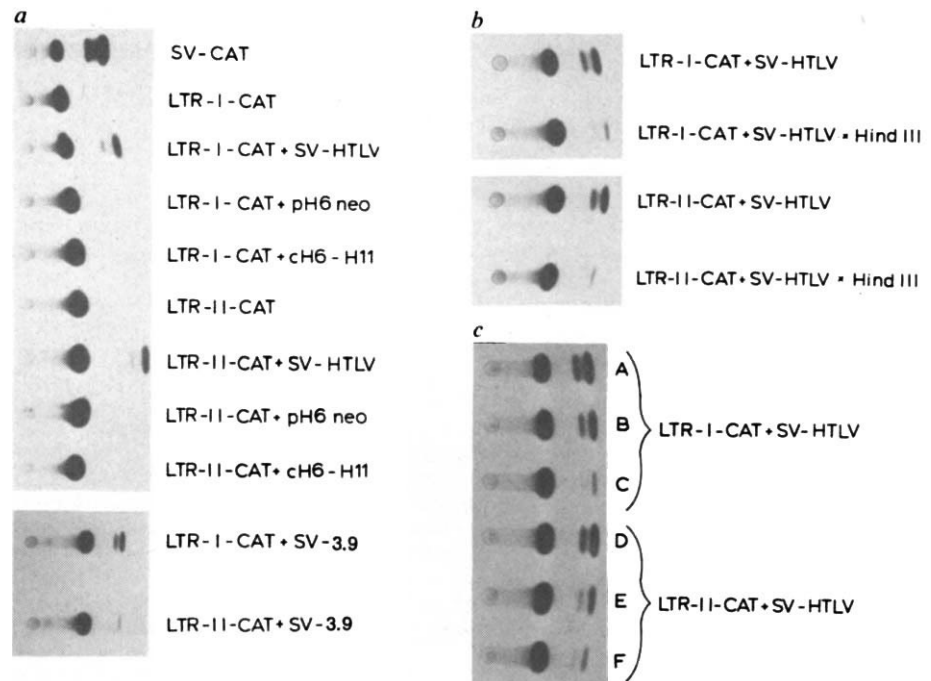


Fig. 1 Schematic structure of constructions used in transfection experiments. Upper line: HTLV-II insert of pH6-neo in the vector pSV2-neo (ref. 34) and cH6-H11 in the vector pSVod (ref. 35). The entire HTLV-II genome (8.9 kb) plus some flanking cellular DNA is present in both constructions. Middle line: structure of construct SV-HTLV, which has an 8.2-kb HTLV-II insert (nucleotide positions 362-8,550) in vector pSV2-neo. The 8.2-kb fragment from a partial *Bam*HI digestion was treated with the large fragment of *Escherichia coli* DNA polymerase I. *Hind*III linkers were added and the fragment was ligated to *Hind*III-digested pSV2-neo. Lower line: structure of construct SV-3.9. A 3.9-kb fragment from the 3' end of the HTLV-I genome (from a *Hind*III site at nucleotide 4,990 to an *Eco*RI site created by *Bal*31 nuclease digestion of HTLV-I proviral DNA and insertion of a synthetic oligonucleotide at position ~8,890) was ligated into pSV2-neo doubly digested with *Hind*III and *Eco*RI. The neomycin resistance and SV40 termination-polyadenylation sequences of pSV2-neo were deleted, and transcription of the HTLV-I insert is terminated in the 3' LTR.

Fig. 2 CAT activity measured in COS cells transfected with HTLV constructions. **a**, High levels of CAT are expressed from a modified construct of the SV40-based vector pSV-CAT³⁶ on transfection into COS cells. The structure of the HTLV-I LTR-CAT construction (LTR-I-CAT) and HTLV-II LTR-CAT (LTR-II-CAT) is as described elsewhere^{15,20}. Transfection with LTR-I-CAT or LTR-II-CAT alone produces a very low background CAT activity. Co-transfection with SV-HTLV produces CAT activity approaching that obtained with pSV-CAT. Co-transfection with constructions pH6-neo or cH6-H11 produces CAT activity not significantly higher than that obtained with LTR-CAT alone. Relative conversion of chloramphenicol into acetylated forms (expressed as a percentage of SV-CAT control) from at least three experiments was as follows: LTR-I-CAT, <1%; LTR-II-CAT, <1%; SV-HTLV+LTR-I-CAT, 16%; SV-HTLV+LTR-II-CAT, 9%; SV-3.9+LTR-I-CAT, 10%; SV-3.9+LTR-II-CAT, <1%. Less than threefold variation in levels of CAT activity observed between assays were not considered significant. **b**, *Hind*III digestion of SV-HTLV cleaves the construct at the ends of the HTLV-II insert. Co-transfection of LTR-CAT with *Hind*III-digested SV-HTLV abolishes (<1% SV-CAT control) the CAT activity seen in co-transfections with the native DNA, indicating that both the SV40 and HTLV-II sequences present in the construction are required to achieve *trans*-activation of LTR-CAT. **c**, Transfection of LTR-I-CAT (5 μ g) with 3.0 μ g, 0.6 μ g and 0.12 μ g of SV-HTLV (lanes A-C); LTR-II-CAT (5 μ g) with 3.0 μ g, 0.6 μ g and 0.12 μ g of SV-HTLV (lanes D-F) of SV-HTLV. pSV2-neo DNA was used as carrier to bring the total amount of DNA to 10 μ g in each case. Transfection with 0 μ g of SV-HTLV induces only a background level of CAT activity (**a**). Dose response to the HTLV-II vector SV-HTLV is similar for both LTR-I-CAT and LTR-II-CAT (see text). **Methods**. 2×10^6 COS cells were seeded on 100-mm plates and transfected after 24 h with 10 μ g of DNA, using the calcium phosphate co-precipitation technique³⁷. Cells were collected 40-48 h after transfection and CAT activity determined by TLC. The amount of CAT activity is indicated by conversion of ¹⁴C-radiolabelled chloramphenicol (left) into acetylated forms (right). Results were quantified by cutting out bands and determining the amount of radiolabel in each by liquid scintillation counting.



tion from the LTR is measured by chloramphenicol acetyl transferase (CAT) activity following transfection of virus-infected cells¹⁶⁻¹⁹. We have used a highly sensitive transient co-transfection system to study activation of the LTR where selection of infected or transformed cells would not affect the results obtained. This system has the further advantage that the effects of individual virus genes can be isolated both from one another and from effects seen in immortalized virus-infected cells.

Figure 1 shows the schematic structure of the HTLV transcriptional units in pH6neo, cH6-H11, SV-HTLV and SV-3.9. In pH6neo and cH6-H11, both of which contain a complete provirus genome from the infectious HTLV-II clone λ H6 (ref. 20), transcription of HTLV-II genes would be expected to occur from the native cap site in the 5' LTR. In SV-HTLV, the HTLV insert is an 8.2-kilobase (kb) *Bam*HI fragment of the virus which extends from the 5' LTR to the 3' LTR but lacks the 5' 361 nucleotides (U3 and part of the R region) and 3' 462 nucleotides (part of R and U5) of the HTLV-II genome. The viral genes are expressed using simian virus 40 (SV40) transcription sequences present in the vector. Removal of the HTLV promoter region from the U3 portion of the 5' LTR and HTLV termination sequences from the 3' LTR results in transcription being promoted by the upstream SV40 promoter-enhancer region and terminated in the SV40 termination-polyadenylation sequences downstream of the HTLV insert. SV-3.9 has a 3.9-kb fragment of HTLV-I which extends from genomic position 4,990 in the *pol* gene to near the 3' end of the provirus genome and encodes all of the sequences necessary for χ gene expression²¹. Transcription is promoted by the upstream SV40 promoter-enhancer region of the vector, as is the case with SV-HTLV, but terminates in the 3' LTR rather than in the SV40 sequences of the vector (Fig. 1).

Figure 2a shows the results of co-transfections in COS cells with each of these constructions and either HTLV-I LTR-CAT (LTR-I-CAT) or HTLV-II LTR-CAT (LTR-II-CAT)^{15,22}. Transcriptional activation of the LTR-CAT constructions occurs

only in co-transfections with the SV-HTLV and SV-3.9 constructs, and not in transfections with the other HTLV constructs. This is due to more efficient transcription of HTLV genes from the SV40 promoter than by the HTLV-II LTR (ref. 20 and see Fig. 2a). The HTLV-II construction SV-HTLV is capable of activating transcription from both the LTR-I-CAT and LTR-II-CAT constructs, but the HTLV-I construct SV-3.9 efficiently activates only LTR-I-CAT. This is consistent with the observations of other investigators who found that the LTR of HTLV-I is activated in HTLV-II-infected cells but that the HTLV-II LTR is only weakly active in HTLV-I-infected cells¹⁶. *Trans*-activation is abolished in co-transfections with *Hind*III-digested SV-HTLV, in which the HTLV-II insert is removed from the SV40 promoter/termination sequences in the vector (Fig. 2b). Figure 2c shows the dose-response obtained by co-transfection of COS cells with LTR-CAT and decreasing amounts of the SV-HTLV construction. Efficient expression of the CAT gene by the LTR is clearly dependent on the presence of the intact SV-HTLV construct in the transfection; dilution or restriction endonuclease digestion of SV-HTLV decreases or abolishes the activation of LTR function. Therefore, transcriptional activation of the LTR is dependent on expression of HTLV functions which are supplied in these co-transfection experiments by SV-HTLV. Similar results were obtained in co-transfections of other cell lines, including HeLa cells and lymphoid cells such as Epstein-Barr virus-transformed B cells (data not shown). We will refer

to this *trans*-acting transcriptional activation of LTR functions as the *trans*-regulatory function of HTLV.

To determine which of the HTLV genes was responsible for activation of the LTR, we introduced mutations into the SV-HTLV construction described above. These mutants were used in the COS cell co-transfection system together with LTR-CAT (see Fig. 3). The HTLV χ gene is encoded by a 2.1-kb messenger RNA derived by the splicing of two introns, the first of which contains the *gag* and *pol* genes and the other the major part of the *env* gene and the untranslated region of the genome²¹. The middle exon supplies four nucleotides which encode the NH₂-terminus of the χ protein. These four nucleotides are derived from the *env* gene methionine codon and one additional nucleotide which is spliced to the major χ open reading frame of the third exon. Only mutations which affect the expression of χ (SV-HTLV-Xho and SV-HTLV-Cla) (see Fig. 3 legend for details of constructs) abolish transcriptional activation. Other mutations in the *gag*, *pol* and *env* genes (SV-HTLV-Sst, Xba, EXB) did not abolish the *trans*-regulatory function of the genome. Note that mutants bearing deletions in the 700-bp nontranslated region of the HTLV-II genome (SV-HTLV-ExB

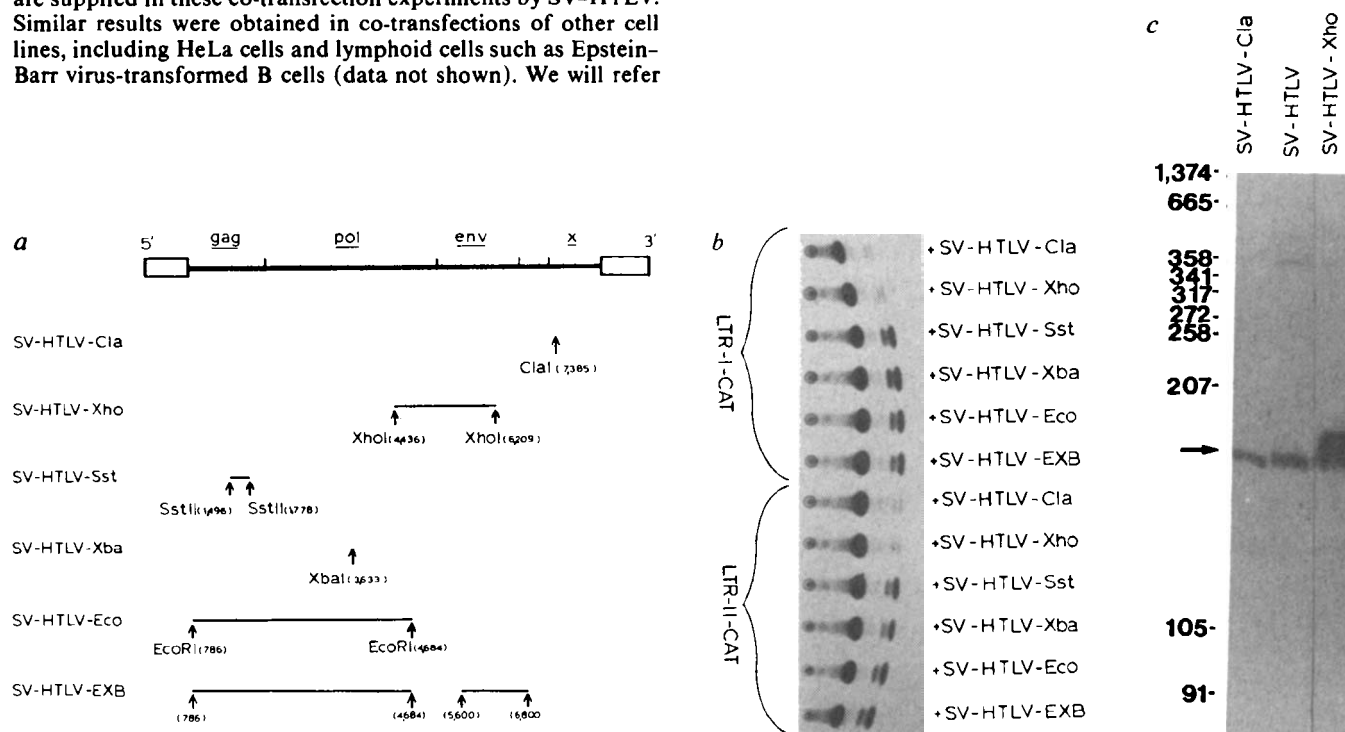


Fig. 3 **a**, Location of mutations introduced into SV-HTLV. The mutant SV-HTLV-Sst has a 309-bp deletion in *gag* p24 (position 1,469–1,778) and activates the LTR in co-transfection assays. SV-HTLV-Xba has a frameshift mutation in the *pol* gene, due to a 4-bp insertion at position 3,633. These four bases were introduced by treatment of *Xba*I-digested SV-HTLV with the large fragment of *E. coli* DNA polymerase I and recircularization with DNA ligase. Although this mutant would be expected to produce a truncated *pol* gene product, this does not affect *trans*-regulation of the LTR. SV-HTLV-Eco has a 3,898-bp deletion (788–4,686), created by removal of an *Eco*RI fragment, which deletes the whole of the *gag* region and most of the *pol* gene but leaves intact *env* and χ gene coding sequences²¹. SV-HTLV-EXB was derived from SV-HTLV-Eco by a *Bal*31 nuclease deletion at a *Xho*I site at position 6,209 in the *env* gene of SV-HTLV-Eco. The *Bal*31-induced deletion in this mutant is ~1.2 kb long and extends from the *env* glycoprotein gp52 at the 5' end into the nontranslated region upstream of the χ open reading frame (position ~5,700–6,900). This mutant has deletions of *gag*, *pol* and *env* which total 5.1 kb of the 8.2-kb HTLV-II insert in SV-HTLV and is still capable of *trans*-activating both LTR-CAT constructions efficiently. SV-HTLV-Xho contains a 1,773-bp deletion (4,436–6,209) in the HTLV insert which deletes the 3' portion of the *pol* gene, *env* glycoprotein gp52 and the 5' portion of the transmembrane protein p20. Although this mutant retains the major coding region of the χ gene in the third exon, it is not expected to produce a functional χ gene product because of the deletion of the middle exon containing the initiation codon required for χ gene expression²¹. Co-transfections with this mutant do not produce increased CAT activity over that seen with LTR-CAT alone. SV-HTLV-Cla has a frameshift mutation in the χ gene, due to a 2-bp insertion into *Clal*-digested SV-HTLV at position 7,385. This change is expected to produce an altered χ protein. The native p37^{HTLV} protein (relative molecular mass 40,000) comprises a total of 339 amino acids, 337 of which are coded between nucleotide positions 7,190 and 8,202 in HTLV-II. The altered predicted protein from SV-HTLV-Cla comprises 158 amino acids, that is, 67 from the native protein at the N-terminus and 91 amino acids from a different reading frame at the C-terminus (position 7,660). This predicted protein, unlike the native protein produced by SV-HTLV, cannot activate LTR-CAT. **b**, CAT activity in COS cells co-transfected with LTR-CAT and SV-HTLV mutants. Cells were transfected and CAT activity measured as described for Fig. 2. Lanes 1–6 received 5 μ g of LTR-I-CAT; lanes 7–12, 5 μ g of LTR-II-CAT. 5 μ g of DNA from each mutant were used as indicated on the figure. Relative conversion of chloramphenicol into acetylated forms (as a percentage of SV-CAT control) from at least three experiments was as follows: LTR-I-CAT+SV-HTLV-Cla, <1; +SV-HTLV-Xho, <1; +SV-HTLV-Sst, 20; +SV-HTLV-Xba, 21; +SV-HTLV-Eco, 10; +SV-HTLV-EXB, 15; LTR-II-CAT+SV-HTLV-Cla, <1; +SV-HTLV-Xho, <1; +SV-HTLV-Sst, 17; +SV-HTLV-Xba, 22; +SV-HTLV-Eco, 11; +SV-HTLV-EXB, 13. Less than threefold variation in levels of CAT activity observed between assays was not considered significant. **c**, S₁ nuclease analysis of 50 μ g of cytoplasmic RNA from transfected COS cells. S₁ analysis of χ mRNA was performed using a *Hha*I/*Clal* ³²P-5' end-labelled probe (nucleotide positions 6,957–7,387) for the HTLV-II χ splice-acceptor site (position 7,214). Processed χ mRNA is indicated by the presence of a protected DNA fragment of 174 nucleotides, indicated by an arrow (see ref. 38).

and others; data not shown) between *env* and χ also retained *trans*-regulatory function, indicating that this region is not involved in expression of *trans*-regulatory functions as assayed by co-transfection.

S₁ nuclease analysis was used to examine the production of χ RNA by the SV-HTLV mutants. An S₁ nuclease probe specific for processed χ mRNA demonstrated that comparable levels of mRNA were produced by each of the constructions including the χ gene mutant SV-HTLV-Cla, indicating that the defect in SV-HTLV-Cla is in the translation of a functional χ gene product, not in χ mRNA levels (Fig. 3c). The apparently aberrant splicing observed with SV-HTLV-Xho may be due to loss of the second exon in the normal χ mRNA. These results demonstrate that only mutations which interfere with correct processing (SV-HTLV-Xho) or translation (SV-HTLV-Cla) of χ gene mRNA prevent *trans*-regulation of LTR-CAT. Other mutations which do not prevent expression of χ , including those that abolish all other viral genes, *gag*, *pol* and *env* (SV-HTLV-EXB), do not affect LTR *trans*-regulation, indicating that expression of the χ protein appears to be the single factor mediating *trans*-regulation and that no other virus gene is required.

HTLV differs from other retroviruses by the presence of a distinct locus, the χ gene, which is responsible for transcriptional regulation. A transcriptional activator locus has also recently been identified in a different reading frame of the *gag* gene of Rous sarcoma virus²³. There appears to be no comparable locus in HTLV, as mutants which completely lack the *gag* gene still activate the LTR efficiently. Unlike the χ gene of HTLV¹⁵, it is not known whether the *gag* activator locus is essential for replication of Rous sarcoma virus. These studies show directly that the requirement of the χ gene for viral replication is due to the transcriptional regulatory functions of the χ gene on the LTR. The presence of the χ gene may provide additional advantages for transcriptional regulation of HTLV expression in the host cell. The HTLV-II construct SV-HTLV can activate both the HTLV-I LTR and HTLV-II LTR, indicating a similar function for the χ proteins of both viruses, consistent with the sequence homology between the HTLV χ proteins^{13,14} and similar biological properties of HTLV-I and -II χ proteins⁵⁻⁸. The failure of the HTLV-I construct SV-3:9 to activate transcription from LTR-II-CAT is probably due to differences in the LTRs of HTLV-I and -II.

The function of the χ gene in HTLV transcription and replica-

tion must have a major influence on cellular transformation by, and on the pathogenicity of, HTLV. The kinetics of HTLV replication following infection may be related to the level of χ gene expression. Following integration into the genome of the host cell, low levels of χ mRNA are transcribed. Production of small amounts of the χ protein would stimulate LTR function, in turn producing higher levels of χ protein, and so on. This hypothesis is consistent with the slow kinetics observed in HTLV infection of cells (J.D.R., unpublished) and contrasts with typical retrovirus infections, where stable levels of RNA expression merely require passage of the infected cell through the S phase of the cell cycle^{24,25}.

Functional analogies exist between the HTLV χ gene and transcriptional regulatory genes of some DNA viruses. The best studied example of such a gene is the *E1a* gene of adenoviruses. Like χ , the product of *E1a* regulates adenovirus transcription by stimulating the transcription of other adenovirus genes^{26,27}. Since adenoviruses are capable of transforming certain cell types and *E1a* itself is required to transform cells^{28,29}, it seems likely that the HTLV χ gene may also be involved in cellular transformation. Structural and functional similarities have recently been found between the cellular oncogenes *myc*, *myb* and adenovirus *E1a* proteins³⁰, and we have recently shown that there are striking functional similarities between χ and *E1a*³¹. It is becoming apparent that the χ gene shares functional similarities with other transcriptional regulatory proteins, and like them, may also be involved in oncogenesis, possibly by causing aberrant transcriptional regulation in cells in which they are expressed (see refs 32, 33). Studies on the similarities between χ and other proteins known to be capable of cellular transformation will help to determine the role of the χ protein itself and the influence of altered transcriptional regulation as a mechanism for carcinogenesis.

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Note added in proof: Since the submission of this manuscript, two additional reports have confirmed the role of the χ gene in transcriptional activation of the HTLV LTR^{39,40}.

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Dissociation of transforming and *trans*-activation functions for bovine papillomavirus type 1

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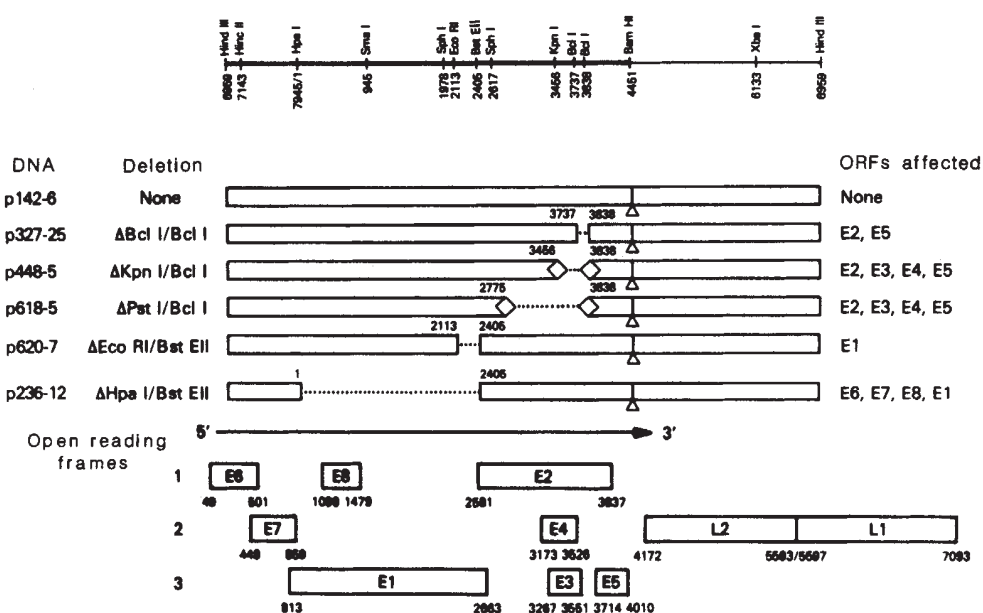
It has been shown that genetic information encoded by the 3' open reading frames (ORFs), E2, E3, E4 and E5, of bovine papillomavirus type 1 (BPV-1), is sufficient to induce cellular transformation of certain mouse cells¹⁻³. The product of the E2 ORF has further been shown to be responsible for the *trans*-activation of a transcriptional regulatory element located in the noncoding region (NCR) of the BPV-1 genome⁴. To examine whether or not the E2 *trans*-activation function is encoded by the same gene that encodes the 3' ORF viral transformation function, we have now analysed the expression of the *trans*-activation function in series of mouse C127 cells transformed by BPV-1 deletion mutants. In addition, using mutated complementary DNA clones generated by the insertion of a premature translational termination linker into different sites of a BPV-1 cDNA clone containing the 3' ORFs intact, we demonstrate that transformation and transcriptional *trans*-activation functions can be dissociated and that they map respectively to the E5 and E2 ORFs.

BPV-1 or its cloned full-length DNA can transform certain rodent cells *in vitro* and the viral DNA persists as an extra-chromosomal multi-copy plasmid in these transformed cells⁷. The genetic organization of BPV-1 has been established from the analysis of the DNA sequence⁸ and the viral RNA transcripts (Fig. 1)⁹⁻¹³. Mutagenesis studies have identified several regions of the BPV-1 genome which influence the expression of the viral transformation functions. Mutations affecting the E2 ORF result in a reduced ability to transform mouse cells, suggesting a role for the putative E2 viral gene product in the transformation process^{2,4,5}. Mutations affecting the E5 ORF also have a marked effect on transformation efficiency^{5,14-16}. The E6 ORF encodes

an independent transforming protein^{3,21} and its function is critical for expression of the fully transformed phenotype². A transcriptional enhancer, which is activated in *trans* by the putative E2 gene product, has been mapped within the 1-kilobase (kb) NCR of the BPV-1 genome⁶.

To examine whether or not the *trans*-activation function of the E2 gene product is linked to the BPV-1 transforming functions of the 3' ORFs, cell lines transformed by different BPV-1 deletion mutants (shown in Fig. 1) were tested for their ability to activate the NCR enhancer. Table 1 lists the cell lines assayed. C127 cells are not transformed and do not contain BPV-1 DNA sequences, and as such serve as a negative control. NS142-6 cells are C127 cells transformed by a plasmid containing the entire BPV-1 genome cloned into the pML2d vector and provide the factor capable of *trans*-activating the NCR enhancer present in p407-1 (ref. 6). The NS620-7 and NS236-12 cell lines have been transformed with BPV-1 deletion mutants that affect the 5' ORFs (E5, E7, E1 and E8). As previously reported, both of these cell lines can provide the *trans*-acting factor to activate p407-1, confirming that the 3' ORFs encode the diffusible *trans*-acting factor⁶. The NS327-25, NS448-5 and NS618-5 cell lines have each been transformed by BPV-1 mutants containing deletions extending various distances upstream from the *Bcl*I site at base 3,838. The p327-25 mutant has a 101-base deletion which affects the carboxy terminus of E2 as well as the 5' terminus of the E5 ORF, upstream of the first E5 AUG methionine codon. This deletion mutant has a transforming activity similar to that of full-length BPV-1 DNA. The mutants p448-5 and p618-5 contain larger deletions which affect the E3 and E4 ORFs as well as the E2 and E5 ORFs. These mutants transform at a level approximately 10% and 1%, respectively, of that of wild-type BPV-1 DNA or of p327-25 DNA². Consistent with previous studies mapping the *trans*-activating factor to the E2 ORF, none of these mutants were able to activate the NCR enhancer in p407-1, despite their varied transformation capability⁶. These data suggest that BPV-1-mediated transformation and a transcriptional activation imparted by the E2 ORF gene product can be unlinked. It is of interest that the activity of the BPV-1 NCR chloramphenicol acetyl transferase (CAT) plasmid p407-1 is higher in those lines transformed by BPV-1 plasmids mutated in the E1 ORF than in the line transformed by the BPV-1

Fig. 1 Structure of the BPV-1 DNA deletion mutants used in transforming mouse C127 cells assessed for providing the NCR/CAT *trans*-acting factor. The recombinant plasmid and deletion are indicated on the left. Each of these plasmids is cloned in pML2d at the *Bam*HI site as indicated by an open triangle. The retained sequences (open box) are covalently linked at the sites indicated and in the figure are separated by the deleted sequences (...). *Xho*I linkers are indicated by a diamond. The ORFs for the BPV-1 genome are indicated at the top of the figure, as are the bases defining the beginning and the ends of each of the ORFs. The E6, E7, E1 and E8 ORFs are referred to in the text as the 5' ORFs because of their location, and the E2, E3, E4 and E5 ORFs are referred to as the 3' ORFs. No significantly large ORFs are located between the *Hind*III site (base 6,959) and the *Hpa*I site (base 7,945/1) and this segment is referred to as the noncoding region (NCR). The construction of each of these deletion mutants has been described previously². The ORFs affected by the deletion mutants are listed on the right. These constructions are presented relative to the map of BPV-1 ORFs and the linear genome. All of the detectable polyadenylated mRNA species are transcribed from a single strand and all ORFs are located on the same strand.



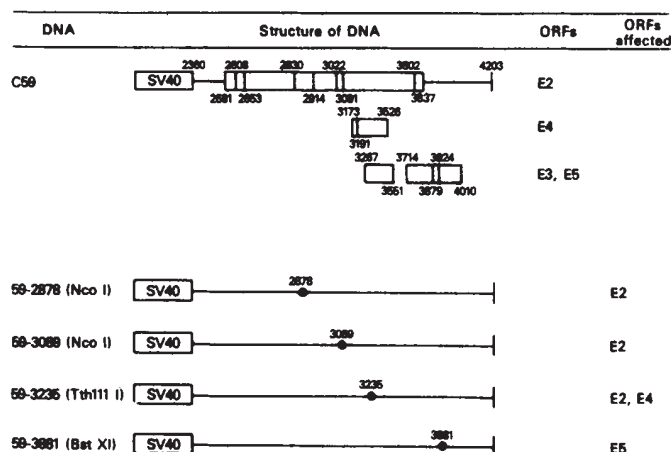


Fig. 2 Structure of the C59 cDNA clone and derived mutants. The structure of this clone is shown at the top of the figure with the SV40 early promoter and late introns located 5' to the BPV-1 ORFs³. The E2, E3, E4 and E5 ORFs are contained intact in the C59 clone. Each of these ORFs is indicated by an open box. Vertical lines in the open boxes represent the AUG codons which could potentially be used to initiate translation. Numbers adjacent to the ORFs represent the 5' and 3' boundaries of the ORFs as well as the location of the AUG codons. The mutants containing the linker fragment with the premature termination codon are indicated below with the position of the in-frame termination codon indicated. The sequence of the synthetic premature translation termination linker which also contains a restriction endonuclease site for *Hpa*I is

TAAGTAACTAG
ATTCAATTGAT

ORFs which would be affected by the insertion of the premature termination codon linker are listed on the right.

full-length genome. Whether this is simply due to experimental cell line variability or to a negative transcriptional regulatory effect mediated by the product(s) of one or more of the 5' ORFs is under investigation.

To separate further the *trans*-activation and transformation functions within the BPV-1 genome, we have introduced a premature translational termination codon linker at various positions into the BPV-1 cDNA clone, C59 (Fig. 2)³. This DNA can transform mouse C127 cells and *trans*-activate the BPV-1 NCR enhancer element^{3,6}. The C59 clone can be predicted to direct the synthesis of the putative E2 protein (relative molecular mass 48,000) with initiation and termination codons at nucleotides 2,608–2,610 and 3,838–3,840, respectively. This cDNA clone, however, also contains the E3, E4 and E5 ORFs intact. By inserting a synthetic linker containing translational stop codons in each of the three reading frames into the different ORFs, we were able to assess the role of each ORF in transformation and *trans*-activation. The structure of each of the resultant linker insertion mutants was verified by DNA sequence analysis and each was shown to contain a single linker insertion (data not shown). Each of the resultant mutants was assayed for its ability to transform mouse C127 cells and to *trans*-activate the NCR transcriptional regulatory element in p407-1 using an acute assay on monkey CV-1 cells (Table 2). The mutants C59-2878

and C59-3089, which affect only the E2 ORF, were unable to provide the diffusible factor for *trans*-activation of the BPV-1/ncr/CAT in the CAT assay; however, they retained the full transforming function on mouse C127 cells. The mutation in 59-3235, located at the *Tth*III site, affects the E2 and E4 ORFs and resulted in the loss of the *trans*-activation function but did not affect the transforming function. Only in 59-3881, in which the premature termination codon was inserted at the *Bst*XI site at base 3,881, was the transformation function abolished. The NCR *trans*-activation function, however, remained intact. The termination linker in 59-3881 is located in the E5 ORF downstream from the first AUG at base 3,879; the E2, E3 and E4 ORFs are intact in this mutant. The retention of *trans*-activation function in 59-3881 indicates that this mutation does not affect the stability of the E2 messenger RNA and strongly suggests that the E5 ORF has a critical role in transformation.

The role of the E5 ORF in transformation has been implicated in other studies using mutants possessing deletions or insertions in the full-length BPV-1 genome^{5,14,16} and in a subgenomic fragment of the BPV-1 genome promoted from a retrovirus long terminal repeat¹⁵. However, these initial studies have not revealed whether the mutations at the *Bst*XI site were altering transformation by affecting critical coding sequences or through regulatory sequences required in *cis*. As yet, no viral RNAs have been found splicing into the E5 ORF and no obvious splice acceptor sequence is located in the vicinity. The fact that the non-transforming mutant 59-3881 provides the *trans*-activation function argues that the mutation at the *Bst*XI site does not affect the stability or translatability of the viral RNA. These results, therefore, provide compelling evidence that the E5 ORF is a coding domain for a critical transforming protein. The E5

Table 1 Chloramphenicol acetyltransferase expression from CAT recombinant plasmids in C127 cells transformed by BPV-1 deletion mutants

Cell line	BPV-1 deletion in transforming DNA	ORFs affected	CAT activity		
			pA ₁₀ CAT	pSV2CAT	p407-1
C127	—	—	0.7	37.5	1.6
NS142-6 I	None	None	0.4	36.5	6.7
NS620-7 B	2,113–2,405	E1	0.6	26.7	17.5
NS236-12 B	1–2,405	E6, E7, E8, E1	0.1	11.2	12.7
NS236-12 C	1–2,405	E6, E7, E8, E1	0.2	42.2	29.2
NS327-25 A	3,737–3,838	E2, E5	0.6	33.7	1.4
NS448-5 A	3,456–3,838	E2, E3, E4, E5	1.1	26.2	3.1
NS618-5 B	2,775–3,838	E3, E3, E4, E5	1.7	29.7	3.3

Each of the cell lines was selected by its transformed phenotype. In each of the cell lines transformed by a BPV-1 deletion mutant, the BPV-1 DNA was in an integrated form². Each cell line was independently transfected with the plasmids pA₁₀CAT²⁶, pSV2CAT²⁷ and p407-1⁶ and assayed 48 h post-transfection for CAT activity. The pSV2CAT plasmid contains the *cat* gene in a modified eukaryotic transcriptional cassette promoted by the full simian virus 40 (SV40) early region promoter including the repeated 72-base-pair functional enhancer²⁷. The pA₁₀CAT plasmid was derived from pSV2CAT and lacks the functional enhancer but still contains the early promoter²⁶. The p407-1 plasmid was derived from pA₁₀CAT by the insertion of the BPV-1 *Hind*III/*Hpa*I NCR fragment in the *Bgl*II site of pA₁₀CAT, 5' to the SV40 early promoter in the transcriptional sense orientation⁶. CAT activity is measured as the % of chloramphenicol which is acetylated by the cellular extract in 30 min at 37 °C. The cellular extract in each assay was normalized for protein content before incubation. Deletions are given in terms of the nucleotide number of the BPV-1 genome.

Table 2 Transformation and *trans*-activation by cDNA clones containing termination codons

Clone no.	Transformation (foci per plate)	CAT activity		
		pA ₁₀ CAT	pSV2CAT	p407-1
C59	26, 40	0.4	61.0	61.0
C59-2878*	21, 31	0.3	55.0	1.7
C59-3089	23, 50	ND	ND	0.3
C59-3235	12, 42	0.3	70.8	1.2
C59-3881	0, 0	0.2	61.2	62.9
Salmon sperm DNA	0, 0	0.3	10.3	1.2

Transformation of C127 cells was carried out with 5 µg of the indicated cDNA clone using salmon sperm DNA as carrier as previously described³. CAT activity is measured as the % of chloramphenicol which is acetylated by the cellular extract in 30 min at 37 °C. CV-1 cells were transfected with each of the above cDNA clones listed and with the plasmids pA₁₀CAT²⁶, pSV2CAT²⁷ and p407-1⁶. Cell extracts were prepared 48 h post-transfection. ND, not determined.

* This clone has been referred to previously as C59N-9 (ref. 6).

ORF would be predicted to encode a 44-amino-acid hydrophobic protein.

The role of the product of the E5 ORF in the pathogenesis of a fibropapilloma is not known, although it may be involved in the proliferation of the dermal fibroblasts characteristic of a fibropapilloma. ORFs analogous to E5 in BPV-1 have been identified in other papillomavirus genomes that have been sequenced. The sequence of E5 ORFs of HPV-1a (ref. 17) and Shope papillomavirus¹⁸, which induce purely epithelial lesions, however, are not homologous to the E5 ORF of BPV-1. HPV-6b is associated with condyloma accuminata in humans and contains an E5a ORF which shares only slight homology with the E5 ORF of BPV-1¹⁹. There is, however, a small ORF in the corresponding region of the deer papillomavirus genome²⁰ and of the BPV-2 genome (W. D. Lancaster, personal communication) which has the potential to encode a peptide strikingly similar in size and amino-acid composition to the BPV-1 E5 ORF. Like BPV-1, the deer papillomavirus and BPV-2 each induces fibropapillomas in their natural host and fibroblastic tumours in hamsters, and readily transforms rodent cells in culture.

The mechanism by which the putative E2 gene product can *trans*-activate the NCR transcriptional regulatory element and its interaction with other viral or cellular genes during the transformation process remains unknown. The dissociation of the *trans*-activation and transformation functions of the 3' ORFs demonstrated here suggests that the E2 gene product may function indirectly through the activation of the NCR transcriptional regulatory element. The activation of the NCR enhancer by the E2 gene product could lead to increased transcription from the upstream promoter and expression of the 5' ORFs including E6, E6/E7 and E1, which have been shown to affect transformation and plasmid maintenance²⁻⁴. However, we have not ruled out the possibility that the E2 ORF gene product may have a direct effect on transformation. Our assay for transformation has been done using the mouse C127 cell line, which is an established cell line. It is possible that the E2 ORF has a direct transformation function but one that must be assayed in a different cell system. Other viral and cellular genes whose products have *trans*-activation properties, including the adenovirus E1a gene²² and the *c-myc* gene²³, have been shown to interact with other transforming genes in transforming primary cells^{24,25}. It is possible that *trans*-activation is only one of several pleiotropic functions of the E2 gene product.

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Forensic application of DNA 'fingerprints'

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Many highly polymorphic minisatellite loci can be detected simultaneously in the human genome by hybridization to probes consisting of tandem repeats of the 'core' sequence¹. The resulting DNA fingerprints produced by Southern blot hybridization are comprised of multiple hypervariable DNA fragments, show somatic and germline stability and are completely specific to an individual^{2,3}. We now show that this technique can be used for forensic purposes; DNA of high relative molecular mass (M_r) can be isolated from 4-yr-old bloodstains and semen stains made on cotton cloth and digested to produce DNA fingerprints suitable for individual identification. Further, sperm nuclei can be separated from vaginal cellular debris, obtained from semen-contaminated vaginal swabs, enabling positive identification of the male donor/suspect. It is envisaged that DNA fingerprinting will revolutionize forensic biology particularly with regard to the identification of rape suspects.

Biological samples for forensic analysis consist mainly of bloodstains or semen stains on cloth or other surfaces (often several days or even weeks old), vaginal swabs taken after an alleged rape, and sometimes hair roots^{4,5}. Current methods for typing blood and semen stains are reviewed by Divall⁶ and Sensabaugh⁷. Typically, a battery of polymorphic protein and blood group marker systems are used (the Metropolitan Police Forensic Science Laboratory, London, currently use ABO, adenylate deaminase (ADA), adenylate kinase (AK), carbonic anhydrase (CA-II), erythrocyte acid phosphatase (EAP), esterase D (EsD), glyoxalase I (GLO), haemoglobin (Hb), peptidase A (Pep A), phosphoglucomutase (PGM₁), Gm, Lewis and rhesus (Rh) for blood analysis). Detection of PGM₁, EAP, ADA and Rh is difficult after 26 weeks^{7,8}; EsD and CAII cannot be reliably detected after 1 month⁷. In contrast, only five systems are at present in common use for identifying genetic markers in semen (ABO, PGM, GLO, Pep A and Lewis) and all must be used with considerable caution because semen samples analysed are often contaminated with vaginal fluid which itself has enzyme and blood group activity. Semen contains proteolytic enzymes which further reduce the amounts of proteins recovered⁹. Furthermore, bacterial activity may be responsible for producing erroneous results, particularly in the identification of ABO blood groups^{10,11}.

DNA fingerprints can only be obtained from high- M_r , undegraded DNA. Previous attempts to isolate DNA from dead or aged material have been reported from 140-yr-old quagga muscle¹² and 2,400-yr-old mummies¹³. In both cases only low- M_r DNA was obtained, and it was therefore necessary to establish whether intact DNA could be isolated from samples which may be typically encountered in forensic laboratories.

Multiple samples were prepared from 11 different donors. Bloodstains and semen stains were prepared by aliquoting fresh material onto clean cloth which was air-dried before storage at ambient temperature and humidity. Hair roots were analysed

Fig. 1 Isolation of high relative molecular mass DNA from dried bloodstains, semen stains and hair roots. Lane 1, fresh blood (60 μ l); lane 2, 6-week-old bloodstain (60 μ l equivalent); lane 3, 20-month-old bloodstain (60 μ l equivalent); lane 4, fresh semen (20 μ l); lane 5, 5-week-old semen stain (20 μ l equivalent); lane 6, degraded sample of 5-yr-old semen stain (40 μ l equivalent); lane 7, fresh hair roots ($\times 15$) (RNA is also clearly visible from hair roots); lane 8, fresh blood (60 μ l); lanes 9–14, control human placental DNA, 0.1 μ g, 0.2 μ g, 0.4 μ g, 0.8 μ g, 1.6 μ g, 3.2 μ g respectively.

Methods. Whole blood, semen, hair roots, bloodstains and semen stains were incubated overnight in 1.5-ml microcentrifuge tubes at 37 °C in 0.01 M Tris-HCl, 0.01 M EDTA, 0.1 M NaCl (pH 8.0) containing 2% SDS, 20 μ g ml⁻¹ proteinase K and 0.039 M DTT. DNA was purified by two phenol/chloroform extractions and precipitated by the addition of 0.1 volume of 2 M sodium acetate and 2.5 volumes of absolute ethanol. The DNA was pelleted by centrifugation at 15,000g for 5 min, washed with 70% ethanol and repelleted. If a residue remained (suggesting contamination by low- M_r compounds), the sample was dissolved in 20 μ l of sterile distilled water and passed down a small spun column (200 μ l Sephadex G-50 contained in a 0.75-ml microcentrifuge tube)¹⁵. Samples were electrophoresed on a 10-cm 0.5% agarose gel at 80 V for 2 h, and DNA was visualized by staining with ethidium bromide.

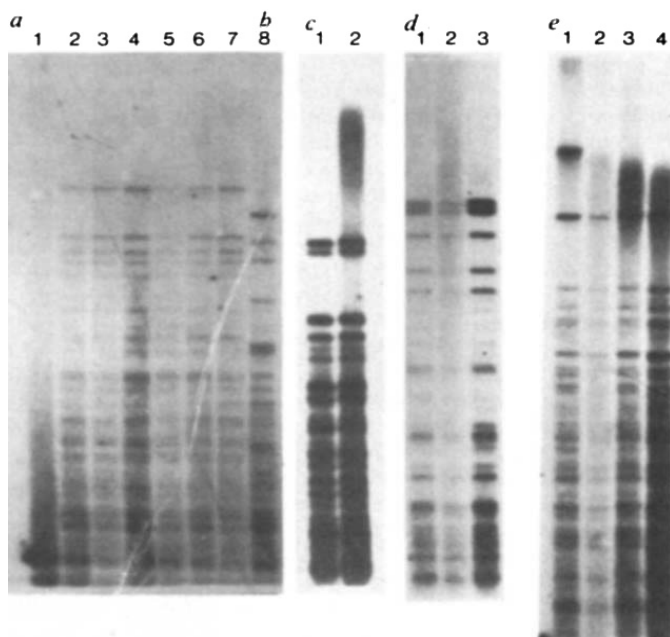
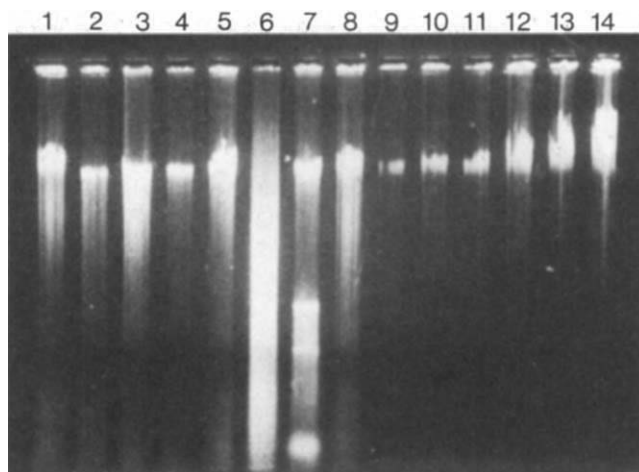


Fig. 2 Somatic stability and ageing studies. Individual *a*: lane 1, degraded sample from 5-yr-old semen stain (40 μ l equivalent); lane 2, 4-week-old semen stain (40 μ l equivalent); lane 3, fresh semen (40 μ l); lane 4, whole blood (60 μ l); lane 5, 4-week-old bloodstain (60 μ l equivalent); lane 6, 2-yr-old bloodstain; lane 7, fresh hair roots ($\times 15$). Individual *b* (showing differences between DNA fingerprints of *a* and an unrelated individual on the same Southern blot): lane 8, fresh blood (60 μ l). Individual *c*: lane 1, fresh semen (20 μ l); lane 2, 11-week-old semen stain (20 μ l equivalent). Individual *d*: lane 1, fresh semen (20 μ l); lane 2, 11-week-old semen stain (20 μ l equivalent); lane 3, hair roots ($\times 13$). Individual *e*: lane 1, fresh blood (60 μ l) (the largest band is high- M_r DNA at the electrophoretic exclusion point); lane 2, 1-day-old bloodstain (80 μ l equivalent); lane 3, 30-month-old bloodstain (80 μ l equivalent); lane 4, 4-year-old bloodstain (80 μ l equivalent).

Methods. Samples were prepared as described for Fig. 1. DNA was digested with *Hinf*I (20 units), in the presence of 4 mM spermidine trichloride, for 2 h at 37 °C. Samples were phenol extracted and ethanol precipitated before electrophoresis through a 20-cm long 0.6% agarose gel at 35 V for 20 h. DNA was denatured *in situ*, transferred to a Schleicher and Schull membrane filter (BA85) and filter-hybridized to ³²P-labelled single-stranded minisatellite probe 33.15 as described elsewhere^{1,2}. Autoradiography was carried out at -80 °C, with an intensifying screen, for 7 days.

fresh. Semen-free and semen-contaminated vaginal swabs (taken at a known period of time after intercourse) were also obtained together with blood samples from both the female and the male. All swabs were stored at -20 °C before use. DNA was extracted from whole blood, whole semen, vaginal fluid, hair roots, bloodstains and semen stains by overnight incubation in an SDS/proteinase K/dithiothreitol (DTT) mixture. Because preliminary experiments showed that semen-contaminated vaginal swabs contained large amounts of DNA from the female, tending to obscure many of the bands from the sperm, female cells were preferentially lysed by preliminary incubation in an SDS/proteinase K mixture. Sperm nuclei are impervious to this treatment because they are ramified with cross-linked thiol-rich proteins¹⁴ and can therefore be separated from the female component by centrifugation. Sperm nuclei were subsequently lysed by treatment with an SDS/proteinase K/DTT mixture. Electrophoresis of samples on 0.5% agarose gels showed that high- M_r DNA could be isolated from bloodstains and semen stains that were at least 2 years old and from fresh hair roots (Fig. 1). However, no DNA could be isolated from hair shafts.

DNA fingerprints obtained from all bloodstains, semen stains and hair roots were shown to be specific to individuals when compared with whole blood and semen samples (Fig. 2). Approximately 5 μ l of semen or equivalent semen stain and 60 μ l of blood or equivalent bloodstain were required. Some samples analysed were partial digests (Fig. 2e) but these also showed patterns consistent with those of a particular individual. DNA fingerprints were obtained from bloodstains up to 4 yr old (Fig. 2e). High- M_r DNA from a 5-yr-old semen stain (Fig. 1, lane 6; Fig. 2a, lane 1) and a 3-yr-old blood stain had degraded so that no pattern was visualized. After using the differential lysis technique only sperm DNA was isolated from a vaginal swab taken 6.5 h after intercourse (Fig. 3, lane 1).

These preliminary results demonstrate that DNA fingerprints are capable of changing completely the emphasis of blood-grouping in forensic science. Using eight polymorphic protein systems together, Sensabaugh⁷ quoted a probability of 0.014 for individual specificity. In practice, the degree of characterization is often much lower, particularly when semen is grouped. This leaves a large degree of uncertainty. In contrast, the condition

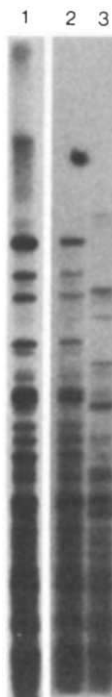


Fig. 3 DNA fingerprint from two vaginal swabs taken 6.5 h after intercourse (lane 1). The pattern is the same as that obtained from the male donor's blood (lane 2). The female's DNA fingerprint (from blood) is shown in lane 3.

Methods. Vaginal cells from semen-contaminated swabs were preferentially lysed by a preliminary incubation in the absence of DTT for 30 min using the lysis mixture described in Fig. 1 legend. Sperm nuclei were pelleted by centrifugation at 15,000g for 5 min, washed once, repelleted and lysed with the full SDS/proteinase K/DTT mixture. DNA was isolated as described in Fig. 1 legend and DNA fingerprints prepared (Fig. 2 legend).

of non-association has been absolute using traditional blood-grouping tests, that is, if the phenotype does not match, a common origin is not possible. Using two DNA minisatellite probes (33.15 and 33.6)¹, the degree of association can approach certainty—thus the probability of chance association using probe 33.15 is $<3 \times 10^{-11}$, and if two probes are used (33.15 and 33.6) the probability is substantially reduced to $<5 \times 10^{-19}$ (ref. 2). In short, using this single method, it is now possible for the forensic scientist to be positive about blood-grouping tests, whereas in the past, it was only possible to be sure of negative associations.

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Light regulation of plant gene expression by an upstream enhancer-like element

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Light regulates many varied physiological and developmental phenomena during plant growth and differentiation, including the formation of a photosynthetically competent chloroplast from a proplastid¹. The expression of ribulose 1,5-bisphosphate carboxylase small subunit (*rbcS*) genes is regulated by light in a development- and tissue-specific manner^{2,3}. In some plant species, phytochrome has been demonstrated to mediate this response^{4,7}, and photoregulation of *rbcS* expression occurs at least in part at the level of transcription^{8,9}. We have shown previously that a 5'-noncoding fragment (4-973 base pairs (bp) upstream of the messenger RNA cap site) of the pea *rbcS* *ss3.6* gene contains all of the nucleotide sequence information necessary to direct the photoregulated expression of a bacterial chloramphenicol acetyltransferase (*cat*) gene in tobacco¹⁰. Consistent with these findings, Morelli *et al.*¹¹ have shown by deletion analysis of a second *rbcS* gene promoter, that the sequences required for photoregulated expression of *rbcS* E9 reside within the 5'-noncoding region. They identified an upstream region of ~700 bp needed for maximum transcription but not light-dark regulation, and a region from -35 to -2 bp which included the TATA box and contained the necessary information for light responsiveness. We now demonstrate that regulatory sequences 5' distal to the *rbcS* *ss3.6* TATA box and transcriptional start site not only contain the information necessary for maximum expression, but also confer photoregulation. These upstream regulatory sequences function independently of orientation when fused to their homologous promoter or a heterologous promoter.

The expression of many animal viral and cellular genes is influenced by regulatory sequences, commonly referred to as enhancers¹²⁻¹⁸, which characteristically function independently of orientation at various distances from the TATA box and start site of transcription^{12,16-18}. Certain enhancers activate gene expression only in specific cell types^{16,18}, or in response to physiological stimuli¹⁵. It has not previously been investigated whether the expression of some plant genes is also regulated by enhancers.

The nucleotide sequence requirement for photoregulated expression of the *rbcS* *ss3.6* gene¹⁹ was examined using the CAT expression system²⁰. We and others^{10,20-23} have demonstrated the ability of this system to compare promoter efficiency and have shown that for chimaeric genes such as those used here, in which the mRNAs are identical, CAT activity reflects the cellular *cat* mRNA levels¹⁰ and therefore the transcriptional activity directed by the targeted promoter. To overcome any bias due to different RNA processing or translation efficiencies, the various test promoter fusions were always compared with the wild-type promoter.

To define more accurately the sequences within the -973 to -4 *rbcS* *ss3.6* promoter which are necessary to direct photoregulated expression, a series of promoter deletion mutants was constructed and tested for their ability to direct photoregulated *cat* gene expression in transformed tobacco calli (Fig. 1). Three of the constructs contain 5' deletions with endpoints at 722, 357 and 92 bp upstream from the cap site and the fourth contains a 3' deletion lacking sequences -4 to -90 (including the TATA box).

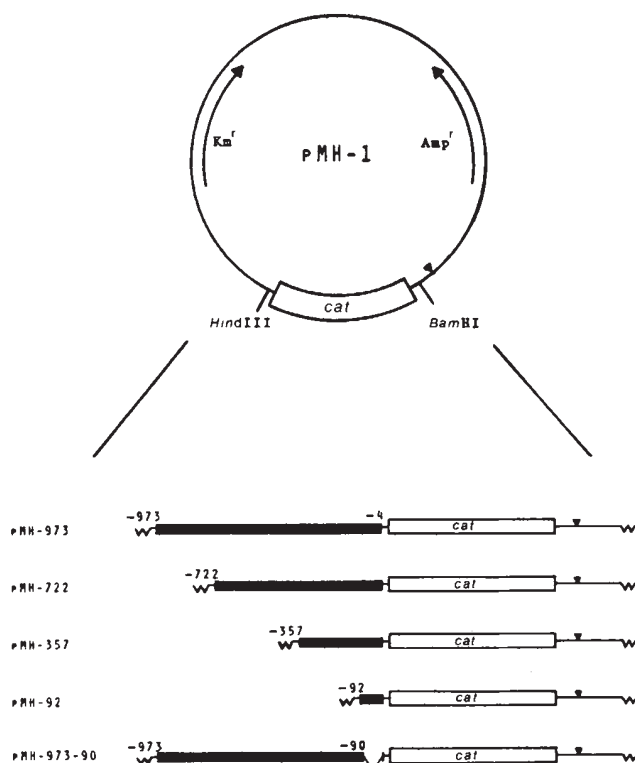
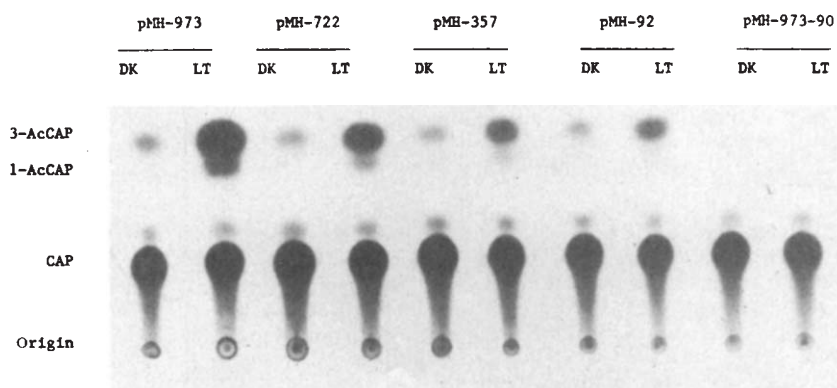


Fig. 2 Chloramphenicol acetyltransferase activity in transformed tobacco calli expressing truncated *ss-cat* chimaeric genes. Chimaeric genes containing truncated *ss3.6* promoters were introduced into tobacco cells and transformed calli grown in light or darkness. The upper panel shows a representative autoradiograph of thin layer chromatographic separation of CAT assay reaction products; the lower panel presents a summary of the data derived from these assays. The plasmid designation for the various deletion mutants appears above each pair of lanes. Extracts from light- and dark-grown tissues are designated LT and DK, respectively. CAP, ^{14}C -chloramphenicol; 1-AcCAP, 1-acetylchloramphenicol; 3-AcCAP, 3-acetylchloramphenicol.

Methods. Plasmids containing truncated *ss-cat* chimaeric genes were mobilized into *Agrobacterium tumefaciens* harbouring either the wild-type Ti plasmid, pTiC58, or the attenuated plasmid, pLGV-3851 (ref. 30), and stable recombinants were used to infect *Nicotiana tabacum* var. SR1 cells¹⁰. Several weeks after inoculation, tumours were removed from the plantlets and axenic homogeneous cell lines of transformed calli selected by hormone-independent growth. Independently derived transformants were subcultured and maintained at 28 °C both in darkness on MS media³¹ containing 3% sucrose and in light (12-h dark/light cycle) on MS media containing 0.1% sucrose and 0.2 $\mu\text{g ml}^{-1}$ 6-benzylaminopurine. Southern hybridization analysis of total DNA from transformed calli was performed to confirm the presence and integrity of introduced chimaeric genes (data not shown). CAT activity was assayed in extracts from transformed calli as described previously¹⁰, except that the reaction mixtures were preincubated at 37 °C for 5 min before the addition of acetyl CoA and the final concentration of acetyl CoA in the reaction was adjusted to 0.3 mM. Reaction products were separated in silica gel plates²¹ and located by autoradiography. Individual reaction products were cut from the silica plate and the radioactivity present determined by liquid scintillation. The amount of ^{14}C -chloramphenicol and its 1- and 3-acetyl derivatives were determined and activity is expressed as % conversion of chloramphenicol into its acetyl derivatives by 100 mg of tissue extract in 30 min. Induction is the ratio of CAT activity in light- versus dark-grown callus as determined from the per cent conversion. To calculate relative activity, levels of induction were compared with those of the -973 *rbcs ss3.6* promoter. The average of at least two to five experiments involving independently derived transformants is presented. The values presented are derived from experiments with cells transformed with chimaeric genes introduced using the *Agrobacterium* vector pGV3851.

Fig. 1 The construction of *ss-cat* chimaeric genes under the control of truncated pea *rbcs ss3.6* promoter fragments. Plasmids encoding truncated *ss-cat* genes are designated by the endpoints of the truncated promoter fragment. Plasmid pMH-973 is equivalent to plasmid pMH-2 (ref. 10). Amp^r, ampicillin resistance; Km^r, kanamycin resistance; □, chloramphenicol acetyltransferase (*cat*) coding sequences; ▽, nopaline synthase (*nos*) polyadenylation signal; ■, *rbcs ss3.6* promoter sequences.

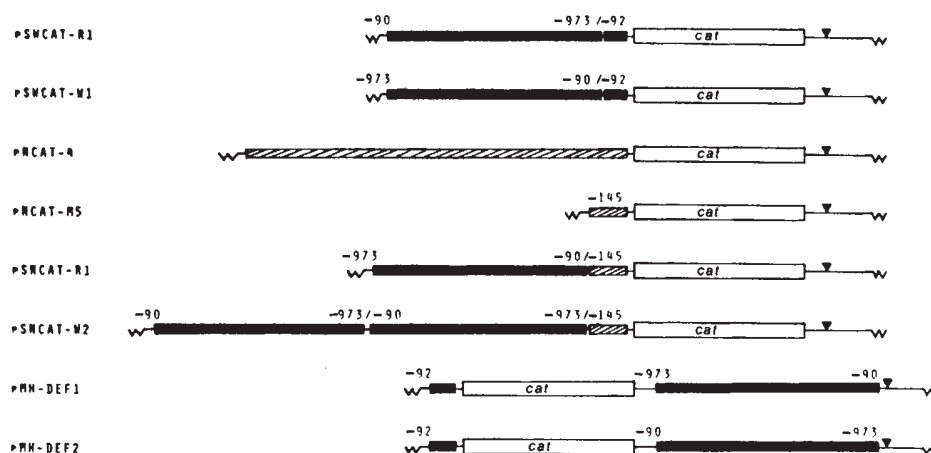
Methods. An ~1-kb *Hind*III restriction fragment containing nucleotide sequences extending from 4 to 973 bp 5' to the mRNA cap site of the pea *ss3.6* gene and 30 bp of pBR327 was isolated from plasmid pPSR6¹⁹ and cloned into the *Hind*III site of pSVO-cat²⁰. To generate 5' deletions, the resulting plasmid was linearized by restriction with *Nde*I and digested with *Bal*31 exonuclease. The ends were repaired with Klenow fragment of DNA polymerase I and the products restricted with *Eco*RI. These fragments were cloned into the *Eco*RI/*Sma*I sites of M13 mp9 and the endpoints of the deletions determined by dideoxy sequence analysis²⁹. Appropriately sized fragments were removed by *Hind*III digestion, isolated by polyacrylamide gel electrophoresis and ligated into the unique *Hind*III site of pMH-1 (ref. 10). To prepare 3' deletions, the *ss3.6* promoter fragment was cloned into pBR322. The resulting plasmid was linearized with *Hind*III and digested with *Bal*31 exonuclease. The digestion products were restricted with *Bam*HI and the ends were filled-in with Klenow fragment of DNA polymerase. The plasmids were recircularized and HB101 transformants screened for regenerated *Bam*HI sites. One such transformant, determined by DNA sequence analysis to contain a deletion within the promoter extending from -4 to -90 5' to the cap site, was selected for further use. This 883-bp truncated promoter fragment was isolated and *Hind*III linkers were attached, then it was cloned into pMH-1.



Plasmid	Growth condition	% Conversion	Induction	Relative activity
pMH-973	Dark	1.1	40.6	1.00
	Light	44.7		
pMH-722	Dark	1.6	8.7	0.21
	Light	13.5		
pMH-357	Dark	1.9	2.9	0.07
	Light	5.5		
pMH-92	Dark	1.8	2.1	0.05
	Light	3.8		
pMH-973-90	Dark	0.1	0.0	0.00
	Light	0.1		

Fig. 3 The structure of chimaeric genes used to examine the effect of orientation and position of the *rbcS* upstream regulatory sequences when fused to their homologous or a heterologous promoter on photo-regulated *cat* gene expression. □, Chloramphenicol acetyltransferase coding sequences; ■, *rbcS* *ss3.6* promoter sequences; ▨, nopaline synthase promoter sequences; ▼, nopaline synthase polyadenylation signal.

Methods. The construction of the various chimaeric genes was as follows. In plasmids pSWCAT-R1 and pSWCAT-W1, the 883-bp fragment (see Fig. 1) containing the *rbcS* *ss3.6* upstream regulatory sequences (–973 to –90 bp) was ligated into the *Hind*III site of pMH-92/39 either in the proper (pSWCAT-W1) or reverse (pSWCAT-R1) orientation to the direction of transcription. Plasmid pMH-92/39 was derived from pMH-92 (Fig. 1) by removing the *Hind*III site 3' to the *rbcS* *ss3.6* TATA box. In plasmids pMH-DEF1 and pMH-DEF2, a single copy of the 883-bp fragment to which *Bam*HI linkers were attached was inserted into the *Bam*HI site 3' to the *cat* coding sequence in pMH-92 (Fig. 1) either in the proper orientation to the direction of transcription (pMH-DEF1), or in the reverse orientation (pMH-DEF2). Chimaeric genes containing *ss-nos* promoter fusions were constructed as follows. Plasmid pNCAT-4²¹ was linearized by restriction with *Sac*II and digested with *Bal*31 exonuclease. The digestion products were restricted with *Hind*III, the ends filled-in with Klenow fragment of DNA polymerase, and the plasmids recircularized with T4 DNA ligase. HB101 transformants were screened for plasmids containing regenerated *Hind*III sites and one such transformant, pNCAT-M5, was determined by DNA sequence analysis³² to contain a truncated promoter extending 145 nucleotides 5' to the cap site. This plasmid was restricted with *Hind*II and the 883-bp *rbcS* upstream regulatory sequences inserted. In plasmid pSNCAT-R1, a single copy of the fragment is present in the proper orientation to the direction of transcription; in pSNCAT-W2 a tandem pair of fragments, both in the reverse orientation, has been inserted.



Expression of the truncated *ss-cat* chimaeric genes was determined by assaying levels of CAT enzyme activity in extracts from each independent dark- and light-grown transformed tobacco callus line (Fig. 2). Chimaeric genes under the control of the –973 promoter directed high levels (~40-fold induction) of photoregulated *cat* gene expression in light- versus dark-grown tissue, while deletion of sequences from –973 to –722 caused a dramatic loss of *cat* gene expression, levels of induction being ~20% of the –973 promoter level. Further deletion of sequences from the 5' end of the promoter resulted in additional loss of promoter strength, but did not abolish light inducibility. Truncated promoter fragments containing only 92 bp upstream from the mRNA cap site still directed photoregulated *cat* gene expression, albeit at greatly reduced levels (Fig. 2, pMH-92). The removal of sequences from –4 to –90, which includes the TATA box, resulted in the complete loss of both light and dark *cat* gene expression (Fig. 2, pMH-973-90). This promoter-less upstream fragment was also unable to direct transcription when cloned in the opposite orientation (data not shown).

Since integration of the T-DNA into the plant genome is a random process, variations in copy number and sites of integration might be expected to result in significant differences in levels of expression among individual chimaeric gene constructs. Estimates of copy number ranged from two to five copies per genome and independent transformants expressing the same chimaeric gene construct did not exhibit more than a twofold variation in levels of *cat* expression irrespective of whether the genes were introduced using pGV3851 or pTiC58 (data not shown) as vector. We prefer to accommodate these small differences by presenting levels of induction determined by comparing CAT activity in light- and dark-grown tissue derived from the same transformant.

To determine whether the upstream regulatory sequences required for high levels of photoregulated expression could act independently of orientation with respect to their homologous promoter, the chimaeric genes contained in plasmids pSWCAT-R1 and pSWCAT-W1 were constructed (Fig. 3). These chimaeric genes contain the *rbcS* *ss3.6* upstream regulatory sequences (an 883-bp fragment extending from –90 to –973 bp 5' to the cap site) fused to the truncated *rbcS* *ss3.6* promoter contained on plasmid pMH-92/39 either in the correct orientation to the

direction of transcription (pSWCAT-W1), or its reverse (pSWCAT-R1). In the correct orientation the fusion promoter essentially recreates the 'wild-type' –973 promoter.

The expression of pSWCAT-R1 and pSWCAT-W1 was assayed in light- and dark-grown transformed tobacco calli (Table 1). Chimaeric genes under the control of the truncated promoter fragments contained on plasmids pMH-92 (Fig. 2, pMH-92) and pMH-92/39 (not shown) exhibited only greatly reduced levels of photoregulated *cat* gene expression. However, chimaeric genes under the control of the promoter fusions contained on either pSWCAT-W1 (correct orientation) or pSWCAT-R1 (reverse orientation) showed approximately equal levels of photoregulated *cat* expression to those of the –973 promoter. Thus, these promoter fusions were capable of restoring wild-type levels of transcriptional activity to the –92 promoter. These observations indicate that *rbcS* *ss3.6* upstream regulatory sequences can direct high levels of photoregulated expression independently of their orientation relative to their homologous promoter.

The ability of the *rbcS* *ss3.6* upstream regulatory sequences to function independently of position relative to the TATA box and transcriptional start site was tested by placing these sequences 3' to the coding region of the *cat* gene under the control of the –92 *ss3.6* promoter (Fig. 3). Plasmids pMH-DEF1 and pMH-DEF2 contain chimaeric genes in which a single copy of the *ss3.6* upstream regulatory sequences has been placed at a 3' location either in the proper (pMH-DEF1) or reverse (pMH-DEF2) orientation to the direction of transcription. In dark-grown calli, the chimaeric genes contained on pMH-DEF1 and pMH-DEF2 exhibited levels of CAT activity similar to those observed for a *cat* gene under the control of the –92 promoter alone (Table 1). When these same constructs were assayed in light-grown calli, there was little or no apparent increase in levels of *cat* gene expression above that directed by the –92 promoter. These data indicate that for the constructs tested, the *ss3.6* upstream regulatory sequences cannot confer high levels of photoregulated expression when placed 3' to the TATA box and transcriptional start site.

These observations are similar to those recently reported for the upstream transcriptional regulatory sequences of yeast *GAL* and *CYC1* genes^{24,25}. These yeast regulatory sequences function

independently of orientation 5' to the TATA box and transcriptional start site, but fail to activate transcription when placed 3'. These observations led to the suggestion that the yeast regulatory sequences may differ mechanistically from other known enhancers²⁵. The *rbcS* upstream regulatory sequences may similarly possess fundamental differences. Alternatively, the potential activity of the *rbcS* upstream regulatory sequences may be blocked by elements residing between them and their site of action when in this 3' location^{12,24-26}.

Since sequences within the -92 *rbcS ss3.6* promoter direct low levels of photoregulated expression independently of additional upstream sequences, it may be argued that the upstream regulatory sequences are not involved in mediating light inducibility, but are involved only in determining absolute levels of expression. To test this directly, we fused the *rbcS ss3.6* upstream regulatory sequences to the normally non-photoresponsive¹⁰ promoter from the nopaline synthase (*nos*) gene²⁷ (Fig. 3). In these fusions a *nos-cat* chimaeric gene (Fig. 3, pNCAT-M5) was used containing a truncated *nos* promoter in which all but 145 bp 5' of the mRNA cap site have been deleted. This truncated promoter retains the 'TATA' and 'CAAT' boxes²⁷ and is capable of directing wild-type levels of *cat* expression in light- and dark-grown transformed tobacco calli (Table 1), consistent with previous observations²⁸.

Table 1 Chloramphenicol acetyltransferase activity in extracts from light- and dark-grown transformed calli expressing chimaeric genes used to test for enhancer-like activity of *rbcS* upstream regulatory sequences

Plasmid	Growth condition	% Conversion	Induction	Relative activity
pSWCAT-R1	Dark	1.4		
	Light	53.0	37.9	0.93
pSWCAT-W1	Dark	1.1		
	Light	41.1	37.2	0.92
pNCAT-4	Dark	5.6		
	Light	4.0	0.0	0.00
pNCAT-M5	Dark	3.9		
	Light	4.4	0.0	0.00
pSNCAT-R1	Dark	3.2		
	Light	53.2	16.6	0.41
pSNCAT-W2	Dark	4.6		
	Light	62.7	13.6	0.33
pMH-DEF1	Dark	1.1		
	Light	5.2	4.7	0.12
pMH-DEF2	Dark	1.2		
	Light	4.6	3.9	0.09

Chloramphenicol acetyltransferase activity was assayed and quantitated as described in Fig. 2 legend. Plasmid designations correspond to the chimaeric genes described in Fig. 3. The values presented are derived from experiments using cells transformed with chimaeric genes introduced using the *Agrobacterium* vector, pGV3851.

The chimaeric gene encoded by pSNCAT-R1 contains a single copy of the *ss3.6* upstream regulatory sequences fused to the truncated *nos* promoter in the proper orientation to the direction of transcription and at approximately the same distance upstream from the *nos* transcriptional control sequences as naturally found in the *ss3.6* promoter. In pSNCAT-W2, a tandem pair of the upstream regulatory sequences, both in reverse orientation to the direction of transcription, are present.

In dark-grown calli, the chimaeric genes encoded in pSNCAT-R1 and pSNCAT-W2 each showed similar levels of *cat* expression to those observed for either the wild-type or truncated *nos-cat* gene (Table 1). This suggests that the *ss3.6* upstream regulatory sequences do not alter dark levels of expression of these genes. As expected¹⁰, on transfer to light neither the wild-type nor truncated *nos-cat* gene showed altered levels of *cat* expression. Chimaeric genes containing the *ss-nos* promoter fusions (pSNCAT-R1 and pSNCAT-W2), however, exhibited

dramatic increases in *cat* gene expression in light- versus dark-grown calli (Table 1). Thus, chimaeric genes containing the *ss-nos* promoter fusions exhibit the same photoregulation observed for the *rbcS ss3.6* promoter. Comparison of *ss-nos* promoter fusions and the -973 *ss3.6* promoter construct revealed that the absolute levels of *cat* expression in the light are similar. However, induction levels for both pSNCAT-R1 and pSNCAT-W2 are ~50% of those observed for the -973 *ss3.6* promoter construct, due to the higher levels of CAT activity in dark-grown calli expressing genes containing the *ss-nos* promoter fusions. Placing the *ss3.6* upstream regulatory sequences inverted and in tandem did not affect the levels of induction when compared with a single correctly oriented copy fused to the truncated *nos* promoter. These results demonstrate that the upstream regulatory sequences can mediate photoregulated expression independently of additional transcriptional signals found within the -92 *rbcS ss3.6* promoter.

We have demonstrated clearly that sequences upstream of the *rbcS ss3.6* TATA box both contain information necessary for maximum levels of expression and confer photoregulation. The *rbcS ss3.6* upstream regulatory sequences function independently of orientation when fused to either their homologous or a heterologous promoter. As these upstream regulatory sequences alone are unable to promote expression when joined directly to the *cat* coding sequence (Fig. 2), we presume that the enhanced expression observed from the fusion promoters reflects an increase in the level of transcripts initiated from the normal start site. Experiments to test this directly are in progress. We conclude that the expression of the plant *rbcS ss3.6* gene is regulated by a genetic element that is similar in many respects to the animal viral and cellular enhancers¹²⁻¹⁸. The ability of the *rbcS* enhancer-like element to function when fused to a heterologous promoter will facilitate its characterization. Moreover, the ability of the *rbcS* and related enhancer-like elements to modulate expression via heterologous promoters is likely to prove useful in the genetic manipulation of plant species.

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Reverse transcriptase activity and Ty RNA are associated with virus-like particles in yeast

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The Ty element of yeast represents a class of eukaryotic transposons that show remarkable structural similarity to retroviral proviruses^{1,2}. Recently, these comparisons have been strengthened by a series of observations on the yeast Ty element: (1) Ty transposes via an RNA intermediate³; (2) it contains a sequence (Fig. 1) which, when translated, is homologous to a conserved region found in all reverse transcriptases^{4,5}; (3) a fusion protein encoded by Ty is produced by a frameshift event that is directly analogous to the production of Pr180^{gag-pol} in a retrovirus such as Rous sarcoma virus^{5,6}. Here we identify the reverse transcriptase activity that, until now, has been presumed to mediate Ty transposition and show that it is sequestered in virus-like particles that also contain Ty RNA.

Most Ty elements are about 5.9 kilobases (kb) long with long terminal repeats (LTRs), called delta (δ) sequences, of ~335 base pairs (bp). The major transcript (5.7 kb long) starts at the *Xho*I site in the 'left' or 5' δ and ends ~50 nucleotides beyond the *Xho*I site in the 'right' or 3' δ ^{7,8}. At the 5' δ -internal sequence junction there is a sequence that could base-pair with the 3' end of a tRNA^{Met} molecule⁹, producing a potential primer-template substrate for reverse transcriptase analogous to that used by retroviruses¹⁰. The transcriptional unit is divided into two open reading frames, designated *TYA* and *TYB*, which overlap in different reading phases by 38 nucleotides^{5,6,11}. *TYA* encodes a protein, designated p1, of relative molecular mass (M_r) ~50,000 (50 K)^{6,12}. *TYB* is expressed, via a frameshift event^{5,6}, as a *TYA*/*TYB* fusion protein (designated p3) of M_r ~190K¹³. Protein p3(Ty1-15) appears to be processed, and a likely product is a 50 K protein designated p2 (ref. 13). The structure and internal organization of the element used in this study, Ty1-15 (ref. 14), is shown in Fig. 1A.

The striking similarities with respect to structure and expression strategy of Ty and retroviruses suggests that the analogy may include the formation of retrovirus-like particles containing Ty RNA; this is apparently the case for the related *copia*-like elements of *Drosophila* which have been reported to produce virus-like particles (VLPs) containing *copia* RNA and reverse transcriptase activity¹⁵. To test this idea for Ty we used the powerful approach of massive overexpression of a Ty transcriptional unit in a high-efficiency yeast expression vector. (We have used this approach previously to identify Ty-encoded proteins^{11,12,13}.) The entire transcriptional unit of Ty1-15 was manipulated to form a convenient *Bam*HI fragment which was then inserted into the *Bgl*II expression site of plasmid pMA91 (refs 16, 17) (Fig. 1B), the resulting plasmid, pMA91-10, drives Ty expression from the promoter of the yeast phosphoglycerate kinase (*PGK*) gene such that Ty1-15 RNA levels are 20-fold higher than that directed by all the endogenous 35 copies of Ty in the genome¹². The construction and authenticity of pMA91-10 have been described previously¹². If Ty produces VLPs there should be many more present in yeast strain T91-10, containing pMA91-10, than in a control strain, T91, containing vector alone. To test this notion, thin sections of the two strains were examined directly in the electron microscope (Fig. 2A, B). In T91-10 there were large numbers of spherical VLPs of ~60 nm diameter

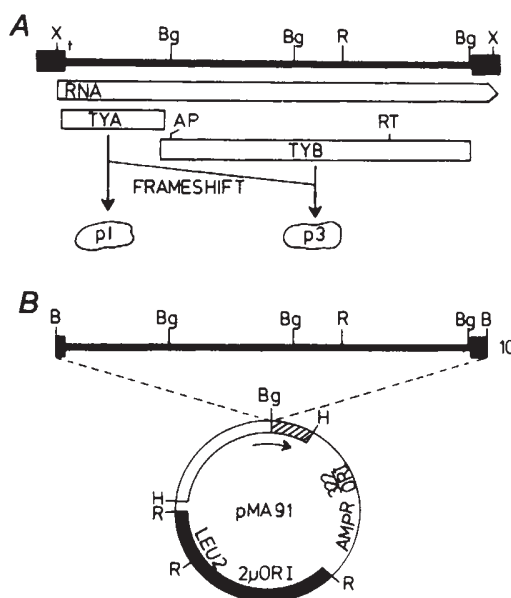


Fig. 1 A, Organization of Ty1-15. Solid boxes, δ sequences. Thick line, internal ϵ region. The major 5.7-kb transcript is indicated. The *TYA* gene extends from nucleotide 299 to 1,619 (ref. 3). The *TYB* gene extends from nucleotide 1,581 to 5,676 (ref. 4 and Fulton *et al.* unpublished data). *TYA* codes for the 50 K protein p1(Ty1-15)^{6,12,13}. *TYB* is expressed as a *TYA*/*TYB* fusion protein of M_r ~190 K, p3(Ty1-15), via a specific frameshift event^{5,6}. t, Region complementary to the 3' end of tRNA^{Met}⁹, AP, region homologous to the active site of acid proteases and retroviral processing proteases³²; RT, region homologous to the conserved region of reverse transcriptases^{4,5}. B, Structure of pMA91-10. The *Xho*I fragment from Ty1-15 was converted to a *Bam*HI fragment (designated fragment 10) as described previously¹² and inserted into the expression vector pMA91 (ref. 16). Plasmid pMA91 is a pBR322 (thin lines) derivative containing a *LEU2*/2 μ origin selection and replication module from pJDB219 (ref. 40) (solid box), the 5' promoter region from the yeast *PGK* gene (open box) and the 3' transcription terminator fragment from the yeast *PGK* gene (hatched box). The *PGK* 5' and 3' regions flank a *Bgl*II expression site. The arrow indicates the direction of transcription promoted by the *PGK* 5' region. X, *Xho*I; Bg, *Bgl*II; R, *Eco*RI; B, *Bam*HI; H, *Hind*III.

whereas in the control strain, T91, there were very few if any. All sections of T91-10 contained particles, which were generally densely packed and represented between ~20% and 80% of the surface area of the sections. The particles appeared to be cytoplasmic and were distributed widely. The difference in numbers of VLPs between the two strains strongly implicates Ty in their formation and we suggest that these particles be designated Ty-VLPs.

If the Ty-VLPs are truly analogous to retroviral particles, they should possess Ty RNA and reverse transcriptase activity⁷. Extracts of T91-10 and T91 were therefore fractionated on 15–45% sucrose gradients and the fractions assayed for reverse transcriptase activity and Ty RNA. Initially the reverse transcriptase assay assumed that there would be an endogenous substrate comprising the Ty RNA with a tRNA^{Met} primer annealed to it via the homology at the δ - ϵ junction⁹, although it should be emphasized that there is no direct evidence for this tRNA acting as a primer in this context. A large peak of putative reverse transcriptase activity was found at a gradient position corresponding to a particulate fraction of ~175 S from T91-10 (Fig. 3A); a smaller peak was obtained from the T91 extract. Coincident with this peak of putative reverse transcriptase activity was a peak of full-length Ty RNA (Fig. 3A), suggesting that, as predicted, the 175 S particles contain both the enzyme

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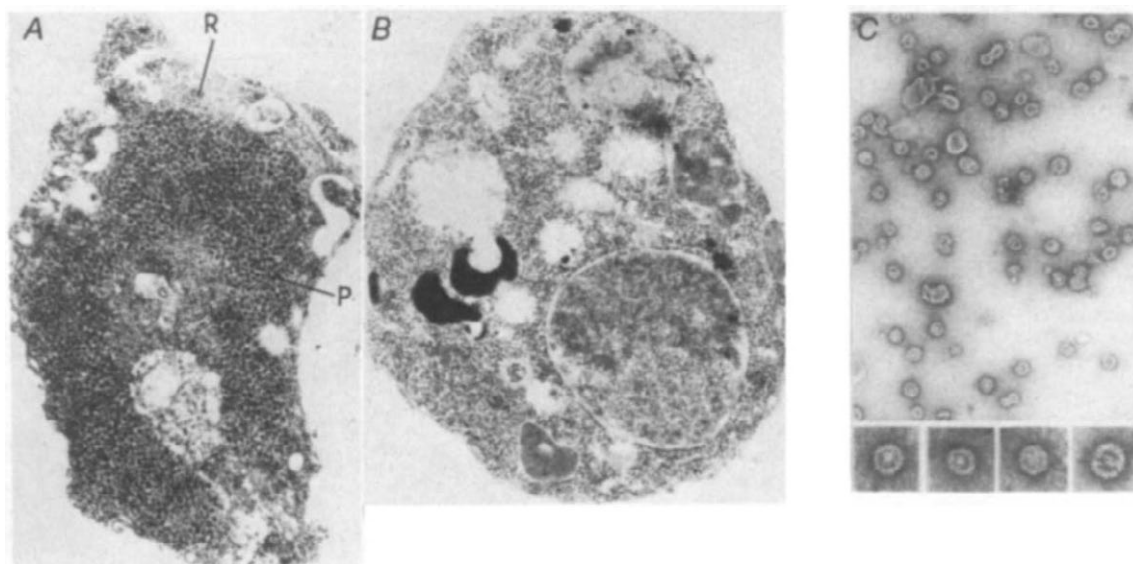
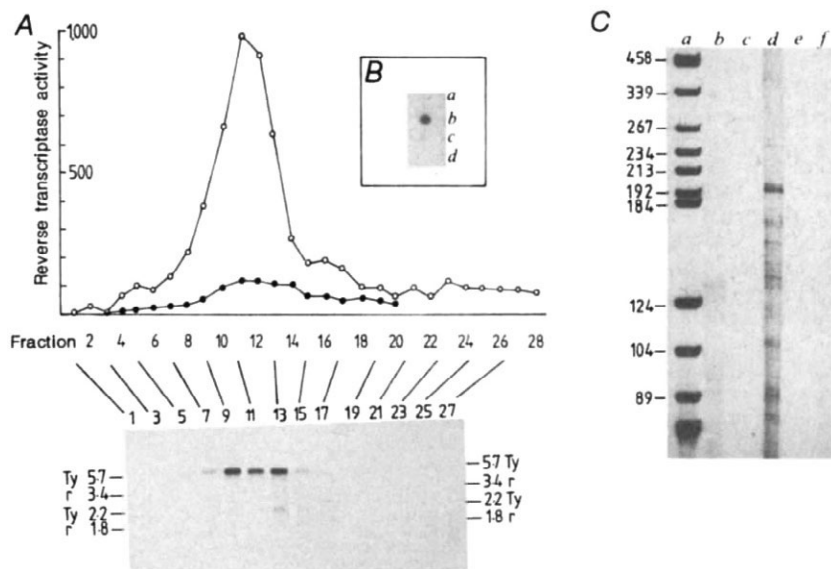


Fig. 2 Electron microscopy of Ty-VLPs. **A**, Thin section of T91-10 ($\times 7,800$). P, Area containing predominantly Ty-VLPs; R, area containing predominantly ribosomes. **B**, Thin section of T91 ($\times 7,800$). Ribosomes can be seen over most of the section. **C**, Uranyl acetate-stained Ty-VLPs from pelleted fractions of a T91-10 extract (low magnification, $\times 30,160$; high magnification, $\times 72,800$).

Methods. Thin sections were prepared according to the method of Byers and Goetsch⁴¹. Purified particles were prepared for electron microscopy by placing 5 μ l of the resuspended pellet on a Formvar/carbon-coated grid and allowing the particles to settle for a few minutes, after which a drop of 2% glutaraldehyde was added, followed by two or three drops of aqueous uranyl acetate. The sections were observed in a Phillips 410 transmission electron microscope.

Fig. 3 **A**, Sucrose gradient fractionation of extracts of T91-10 and T91. The plot shows reverse transcriptase activity in c.p.m. bound to DE81 paper (see below) in fractions from 15–45% sucrose gradients containing material from T91-10 ($\circ$) and T91 ($\bullet$). Below the profiles are shown autoradiographs of Northern blots of RNA isolated from the fractions, bound to nitrocellulose and then probed with nick-translated^{1,42} Ty DNA. The positions and sizes of two ribosomal RNA markers (r) and two Ty RNAs (Ty) are marked. **B**, Dot-blot analysis of reaction products. Reverse transcriptase reaction products were hybridized to four DNA spots: a, no DNA; b, Ty DNA (pAT153-11, which comprises pAT153 containing the left-most *Xho*I–*Bgl*III fragment of Ty1–15; see Fig. 1); c, rDNA (pYRG12)⁴³; d, pAT153 DNA⁴⁴. **C**, Labelled endogenous reaction products separated on a sequencing gel: a, M, markers; b, reaction with low concentration (1 μ M) of cold dNTPs; c, ³²P-labelled dTTP replaced by ³²P-labelled UTP; d, standard reaction (100 μ M cold dNTPs); e, standard reaction followed by treatment with DNase I (50 μ g ml⁻¹; Worthington); f, reaction with no cold dNTPs.



Methods. VLPs were isolated as follows. Yeast cells were grown selectively at 30 °C to a density of $5\text{--}8 \times 10^6$ cells ml⁻¹. The cells were then collected by low-speed centrifugation, washed once in water and resuspended in TEN buffer (10 mM Tris pH 7.2, 2 mM EDTA, 100 mM NaCl) at 1 ml per 1 cells. The cells were disrupted by vortexing with glass beads (40-mesh; BDH) at 4 °C until >70% were broken, then the supernatant was removed and sonicated 10 times on ice for 15 s (6–7 μ m peak-to-peak). The debris was removed by centrifugation (40,000 g for 1 h at 4 °C), and the supernatant layered onto a 15–45% (w/v) sucrose gradient in 10 mM Tris pH 7.2, 10 mM NaCl and spun at 76,300 g for 3 h at 15 °C. Fractions were collected through the bottom of the tube and assayed for reverse transcriptase activity. The endogenous substrate assay was an adaptation of the method of Goff *et al.*⁴⁵. 10 μ l of sample, usually an undiluted sucrose fraction, was added to 20 μ l of an assay cocktail to give the following reaction concentrations (in mM): 50 Tris-HCl pH 8.3, 20 dithiothreitol, 0.6 MnCl₂, 2 MgCl₂, 60 NaCl, 0.1 dATP, 0.1 dCTP, 0.1 dGTP, and 0.06 U ml⁻¹ RNasin (P & S Biochemicals), 0.05% NP40, 0.02 μ Ci μ l⁻¹ [α -³²P] TTP (400 Ci mmol⁻¹; Amersham). This mixture was incubated at 20 °C for 2 h, after which 15 μ l of each assay was transferred to a DE81 filter (Whatman). The filters were washed three times in 5% Na₂HPO₄ for 15 min, once in water, twice in ethanol, then allowed to air-dry. The filters were then counted in a scintillation counter. Products of the endogenous substrate were analysed as follows. ³²P-labelled products were extracted twice with phenol/chloroform (1:1) in TEN, 0.1% SDS and precipitated with 2.5 vol. ethanol, 0.2 M potassium acetate at –70 °C. The precipitate was resuspended in hybridization buffer, added to the DNA filters (see below), incubated overnight at 42 °C and washed twice for 1 h in 2 $\times$ SSC, 20 mM PO₄, 0.1% SDS. Filters were exposed to film at –70 °C with two intensifying screens. RNA isolation and Northern blotting were carried out as described previously¹². DNA was dot-blotted onto nitrocellulose according to Kafatos *et al.*⁴⁶. Detailed analyses (see Figs 2 and 4) were carried out on particles that had been repurified on a second sucrose gradient (25–45%) and then pelleted at 100,000g. S values for the particles in the peak fractions were obtained by re-running the fraction in a Beckman Model E analytical ultracentrifuge.

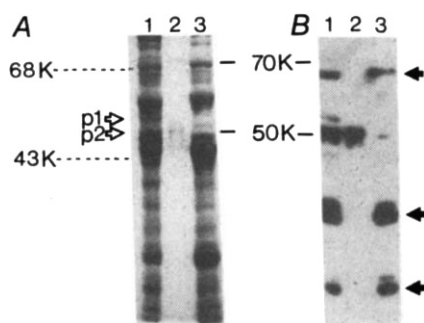


Fig. 4 Ty-VLP proteins, **A**, Coomassie blue-stained proteins from T91-10 (lane 1), isolated Ty-VLPs (lane 2) and T91 (lane 3). The p1 and p2 proteins in T91-10 and the 70K and 50K proteins from the Ty-VLPs are marked. **B**, DNA-binding profiles of the proteins in **A** electroblotted onto nitrocellulose and probed with 32 P-labelled yeast DNA. Lanes 1–3 same as for **A**. The arrows mark the three major dsDNA-binding proteins present in yeast extracts.

Methods. VLPs were isolated as described in Fig. 3 legend. Pelleted gradient fractions (see Fig. 3 legend) were diluted fivefold with 10 mM Tris pH 7.5, 10 mM NaCl and precipitated for 2 h at -70°C after the addition of 2 vol. cold acetone and NaCl to a final concentration of 0.1 M. The precipitates were resuspended in Laemmli buffer⁴⁷, boiled for 5 min and loaded onto a 9% acrylamide/bis (30:1.6) gel with a 5% stack. The gel was stained with Coomassie blue. DNA-binding assays were performed as described previously¹³ and followed a procedure similar to that described by Bowen *et al.*⁴⁸. Nick-translated⁴² total yeast DNA was used in the assay.

and its template. In addition to the full-length RNA, a 2.2-kb Ty RNA was present in the particles. A Ty RNA of approximately this size has been reported by Taguchi *et al.*¹⁸ but its function is unknown. The peak fractions (10–13 in Fig. 3A) were pooled and examined in the electron microscope (Fig. 2C); Ty-VLPs like those seen in the thin sections were present in the pooled fractions. The particles were relatively pure, the major contaminant being membrane fragments. They appear to be composed of a central core and an outer shell. There were no such particles in fractions 5 and 18.

The putative reverse transcriptase activity was characterized in various ways. The labelled products of the endogenous assay were run on a sequencing gel and shown to be a heterogeneous collection of fragments a few hundred nucleotides long (Fig. 3C). These products, which disappeared totally after treatment with DNase, were substantially reduced if a 100-fold lower concentration of dNTPs was used, and were absent if no dNTPs were added to the reaction. Furthermore, no such products were seen when 32 P-UTP was used as the labelled species. It is likely, therefore, that these products are DNA derived from a sub-optimal reaction. In a dot-blot hybridization experiment which compared the hybridization of the products to Ty, ribosomal and plasmid pAT153 DNA, the products hybridized predominantly to the Ty DNA dot (Fig. 3B). The low degree of hybridization to the ribosomal dot was due to small amounts of contaminating ribosomal RNA throughout the gradient (data not shown). These data show that the activity in the peak fractions incorporates deoxynucleotides preferentially into Ty DNA. Table 1 lists some of the properties of this activity. Incorporation in the endogenous assay is reduced by 76% after pretreatment with RNase, suggesting that a component of the endogenous substrate is RNA; that the activity is only partially sensitive to RNase may be due to protection of the RNA template by the particles. Similar results have been obtained by others^{15,19–21}. An activity profile similar to that of the endogenous assay (Fig. 3A) was also seen with poly(rC)-oligo(dG), poly(rCm)[poly(2'-O-methylcytidylate)]-oligo(dG) and poly(rA)-oligo(dT) primer-template substrates; peak activities for these are shown in Table 1. Poly(rC)-oligo(dG) is a poor substrate for DNA-dependent DNA polymerases, while

poly(rCm)-oligo(dG) is not used at all as a substrate for these enzymes^{22,23}. Furthermore, the endogenous reaction is resistant to treatment with actinomycin D, which inhibits DNA-dependent polymerases. These results suggest strongly that the activity associated with the Ty-VLPs is not a DNA-dependent DNA polymerase. In the poly(rC)-oligo(dG) assay the activity is dependent on both primer and template and does not incorporate significant amounts of 32 P-TTP. These data show that the incorporation of label does not occur via some nucleotide transferase activity. Taken together, these observations strongly suggest that the activity associated with Ty-VLPs is a reverse transcriptase.

Retroviral reverse transcriptase activity is dependent on the presence of divalent cations. Different enzymes show different optima for Mn^{2+} and Mg^{2+} (ref. 24), similarly, the activity associated with Ty-VLPs is totally dependent on the presence of either Mn^{2+} or Mg^{2+} . Retroviral reverse transcriptases are usually liberated from particles by disrupting the viral envelope with detergent²⁰, therefore the activity of the retroviral enzymes *in vitro* is dependent on detergent treatment. When Nonidet P-40 (NP40) was omitted from the Ty endogenous assay there was a reduction of activity of only ~50%. This difference between the Ty-VLP and retroviral systems may be due to the absence of an envelope structure in Ty-VLPs (see below). The Ty-VLP enzyme has a temperature optimum of $\sim 20^{\circ}\text{C}$ although there is not a marked peak of activity; this is compatible with the observation that Ty transposition occurs most frequently at about this temperature²⁵. A detailed comparison of retroviral and Ty enzymes awaits purification of the Ty enzyme and the definition of conditions that reproduce the *in vivo* activity *in vitro*.

As a first step towards the analysis of the protein composition of these Ty-VLPs, we set out to determine whether the particles contained proteins that we had previously recognized as being Ty-specified^{6,12,13}. Peak fractions from the gradients were pooled, repurified on a second sucrose gradient (25–45%) and concentrated by pelleting the particles. The dissociated particle proteins were then run on SDS-polyacrylamide gel electrophoresis (PAGE) alongside a yeast extract known to contain high levels of Ty proteins¹³. Two major protein species of ~ 70 K and ~ 50 K are just visible in the stained gel profiles (Fig. 4A). The rather broad 50 K band co-migrates with the previously identified p2 protein in the T91-10 extracts. Protein p2 is one of the few yeast proteins that binds double-stranded (ds) DNA in a blot-binding assay¹³. Therefore, we asked whether the 50 K protein from the purified particles could bind dsDNA. Figure 4B shows that the p2 DNA-binding band that is present

Table 1 Properties of the Ty-VLP reverse transcription

Assay	Variation	c.p.m. incorporated	Activity
Endogenous	– Mn – Mg	2,249	100
	– NP40	50	2.2
	+ Actinomycin D	1,086	48.2
	+ RNase	2,259	100.4
	Incubation at 10°C	547	24.3
	Incubation at 30°C	1,725	76.7
	Incubation at 37°C	1,927	85.6
	Incubation at 42°C	1,443	64.1
Poly(rC)-oligo(dG)		1,041	46.2
		4,188	100
	– 32 P-GTP + 32 P-TTP	56	1.3
	– poly(rC)	132	3.2
Poly(rCm)-oligo(dG)	– oligo(dG)	172	4.1
		2,505	100
	– 32 P-GTP + 32 P-TTP	41	1.6
Poly(rA)-oligo(dT)	–	2,140	100

The endogenous substrate assay is described in Fig. 3 legend. Additions to the endogenous assay were actinomycin D ($100\ \mu\text{g}\ \text{ml}^{-1}$) and RNase A ($100\ \mu\text{g}\ \text{ml}^{-1}$). Exogenous substrate reactions were the same as the endogenous assay except that either $10\ \mu\text{g}$ of poly(rA) (Calbiochem) and $0.1\ \mu\text{g}$ of oligo(dT) (Collaborative Research) or $10\ \mu\text{g}$ of poly(rC) (Calbiochem) and $0.1\ \mu\text{g}$ of oligo(dG) (Collaborative Research) or $2\ \mu\text{g}$ of poly(rCm)-oligo(dG) (1:1 molar nucleotide ratio) (P-L Biochemicals) were present in each assay instead of the cold deoxynucleoside triphosphates. 32 P-GTP was used instead of 32 P-TTP in the G-C reactions.

in T91-10 but absent from T91 co-migrates with a band from the purified particles. These data strongly suggest, therefore, that the Ty-VLPs contain p2. The nature of the 70 K protein, however, is unclear.

The discovery of reverse transcriptase associated with a VLP that contains Ty RNA provides the first protein components in a mechanistic model of Ty transposition. Presumably, as is the case for retroviruses, the enzyme catalyses the production of the dsDNA form of the element from the full-length particle-bound RNA template and tRNA^{Met} primer, and then integration could be mediated by an endonuclease. In retroviruses this endonuclease derives from a post-translational cleavage of the primary *pol* product²⁶⁻³¹; it remains to be seen whether the same is true for Ty. Irrespective of the enzymological details, it must be established whether the Ty-VLPs act as units of transposition or are simply the result of an obsolete viral packaging pathway that is perhaps conserved to sequester the potentially chaotic reverse transcriptase away from cellular RNAs.

Recent observations^{3-6,32}, and the data presented here, appear to establish a close relationship between Ty and retroviruses. However, a comparison of the genomic organization of Ty with that of a generalized retrovirus reveals a major difference. Most retroviruses contain three major protein-coding regions: *gag*, which codes for the viral core components; *pol*, which encodes the reverse transcriptase and integrative endonuclease; and *env*, which codes for viral surface components². *TYA* and *TYB* correspond, in position, to *gag* and *pol* respectively, but Ty appears to lack an *env* region. Thus, Ty may be considered a 'defective retrovirus' and its lack of *env* may explain its relatively small particle size (60 nm as opposed to 80-120 nm for retroviruses³³), *env* may have been lost because it was not needed after some ancestral retrovirus found itself trapped within the walled yeast cell. Alternatively, it may not have evolved because selection for such a structure would not have occurred in a particle without an extracellular stage.

Intracellular retrovirus-like particles have been identified in other systems. The most notable are the A-type particles seen in mouse cells³⁴ and the VLPs that contain *cop* RNA and reverse transcriptase activity in *Drosophila*¹⁵. It seems feasible, therefore, that the distribution of this class of genetic elements may be as broad as that of authentic retroviruses. These elements may be responsible for a wide range of genomic rearrangements, gene activation phenomena and the generation of various reverse-transcribed products such as intronless pseudogenes³⁵⁻³⁷ and the human *Alu* repeats³⁸.

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Following submission of this paper, similar results were published by Garfinkel *et al.*³⁹.

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Errata

Electrostatics revindicated classically

R. Nityananda

Nature **316**, 301 (1985).

IN line 16 of this item the word 'contribution' should read 'contradiction'.

Labelling the axes of graphs...

L. Wilson

Nature **316**, 489 (1985).

IN this Scientific Correspondence contribution, the third paragraph should begin: 'The example quoted above requires the observation that if "Radioactivity = 5×10^3 c.p.m." then " $5 = (\text{Radioactivity} \times 10^{-3})/\text{c.p.m.}$ " or " $5 = 10^{-3} (\text{Radioactivity}/\text{c.p.m.})$ " or " $5 = \text{Radioactivity}/(\text{c.p.m.} \times 10^3)$ ". Thus the right-hand sides of any of these last three equations ...'

Corrigenda

Hepatitis B virus contains pre-S gene-encoded domains

A. R. Neurath, S. B. H. Kent, N. Strick, P. Taylor & C. E. Stephens
Nature **315**, 154-156 (1985).

IN Fig. 1 legend there is an error in the one-letter-code sequence of peptide pre-S(12-145) in the text to panels A/B. Position 136 should be a valine residue (V) instead of a tyrosine residue (Y).

A crucial epileptogenic site in the deep prepiriform cortex

S. Piredda & K. Gale

Nature **317**, 623-625 (1985).

THE authors' address was incomplete and should read: Department of Pharmacology, Schools of Medicine and Dentistry, Georgetown University, Washington, DC 20007, USA.

Hypervariable telomeric sequences from the human sex chromosome are pseudoautosomal

H. J. Cooke, W. R. A. Brown & G. A. Rappold

Nature **317**, 687-692 (1985).

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